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Proteolytic Cleavages of Molecules Involved in Antigen Processing and Presentation: A Thesis

Lawrence James Thomas
University of Massachusetts Medical School

PROTEOLYTIC CLEAVAGES OF MOLECULES INVOLVED IN ANTIGEN PROCESSING AND PRESENTATION

A THESIS PRESENTED

BY

LAWRENCE JAMES THOMAS

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Pharmacology

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A Thesis

By

LAWRENCE JAMES THOMAS

Approved as to style and content by:

_____
Martin Marinus, Chair of Committee

_____
Neal C. Brown, Member of Committee

_____
Michael W. Fanger, Member of Committee

_____
John E. Mole, Member of Committee

_____
Bruce Woda, Member of Committee

_____
Robert E. Humphreys, Thesis Advisor

_____
Thomas B. Miller, Jr., Dean of Graduate School of Biomedical Sciences

Department Of Pharmacology

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DEDICATION

This thesis is dedicated to my parents

Virginia R. and Andrew H. Thomas.

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PROTEOLYTIC CLEAVAGES OF MOLECULES INVOLVED IN ANTIGEN PROCESSING AND PRESENTATION

August 1989

Lawrence J. Thomas

B.A., Brandeis University

A.A., Quinsigamond Community College

Ph.D., University of Massachusetts Medical School

Directed by: Robert E. Humphreys, M.D., Ph.D.

ABSTRACT

The overall goal of my thesis research was to understand better the mechanisms that control antigen processing and presentation by class II MHC molecules. Towards this goal I investigated ways in which the physical structure and post-translational modifications of the class II MHC alpha and beta chains and associated molecules might serve to regulate antigen processing and presentation. Specifically, I investigated (1) a hypothesis that I_i might aid binding of foreign antigenic peptides to the class II MHC foreign antigen binding site (desetope), and the application of this hypothesis to the prediction of class II-presented peptides; (2) the proteolytic cleavage of I_i to p25; (3) the proteolytic cleavage of the class II MHC alpha and beta chains, and (4) the phosphorylation of I_i and the alpha and beta chains.

In exploring the hypothesis that amphipathic alpha helical peptides digested from foreign antigen, bind to the class II MHC desetope, to be presented to T cell receptors, we found such an extended, amphipathic helix in I_i (Phe_{146}-Val_{164}). A hypothesis was developed that this amphipathic alpha helix of I_i bound to the desetope of class II MHC molecules, and remained there from time of synthesis until catalyzing the charging of the desetope with a foreign peptide. This region of I_i could then be considered to be the prototypic T cell-presented peptide and the "strip-of-helix" algorithm was developed to search the sequences of proteins for similar amphipathic alpha helices. Such peptides might bind to the class II MHC desetope and have a high probability to be presented to the T cell.

The strip-of-helix algorithm calculated the mean hydrophobicity (from Kyte-Doolittle values; Kyte and Doolittle, 1982) of sets of amino acids in axial strips down sides of helices for 3 to 6 turns, at positions n, n+4, n+7, n+11, n+14, and n+18. Peptides correlating well with T cell responsiveness had: (1) 12 to 19 amino acids (4-6 turns of an alpha helix), (2) a strip with highly hydrophobic residues, (3) adjacent, moderately hydrophilic strips, and (4) no prolines to break the helix. This algorithm predicted 10 of 12 T cell-presented peptides in 7 well-studied proteins.

In a study of the post-translational modifications of I_i, an early proteolytic pathway of the destruction of I_i, resulting in the generation of p25, was described. This 25,000 dalton protein, seen in immunoprecipitates with antibodies to class II MHC molecules or to

I_i, was shown to be a C-terminal fragment of a high mannose form of I_i. The evidence for this conclusion includes the following results. [^{35}S]methionine-labeled I_i and associated molecules were immunoprecipitated, denatured, resolubilized and subjected to a second immunoprecipitation with various antibodies. Two antisera to C-terminal peptides of I_i (183-193 and 192-211), but not an antiserum to an N-terminal peptide (12-28), immunoprecipitated p25. A monoclonal antibody (mAb) to I_i immunoprecipitated [^{35}S]methionine-labeled p25 but not [^{35}S]cysteine-labeled p25, consistent with the loss of a portion of I_i containing the only cysteine in I_i, Cys_{28}. [^{35}S]methionine pulse-chase labeling demonstrated the maximal appearance of p25 at 20-40 min chase times. p25 molecules were reduced to about 10.5 kD by treatment with endoglycosidases F and H. p25 was, therefore, generated from a high mannose form of I_i in the ER or cis-Golgi. This finding could either implicate that site for class II MHC desetope charging with foreign peptides or reflect a mechanism for degradation of "excess" I_i molecules in the ER. Digestion of class II MHC antigen-I_i complexes with various proteases yielded fragments, migrating at and near p25 in 2-D electrophoretic gels, which were relatively resistant to further digestion. This observation was consistent with the presence of relatively protease-resistant secondary structures (domains) and a relatively protease-sensitive (IgG hinge-like) region in I_i near its insertion into the membrane.

In a study of the post-translational modifications of the class II MHC alpha and beta chains, well conserved pairs of basic amino acids

in the sequences of these molecules were observed. It was hypothesized these could be sites for proteolytic cleavage, as precedented in other systems (*i.e.* proinsulin processing). These potential cleavage sites fall in significant locations with respect to the deduced structure of the class II MHC desetope, supporting the hypothesis that these cleavages might either aid or destroy antigen presenting functions. To test this hypothesis we looked for remnant polypeptides of the alpha and beta chains. Polypeptides were observed in gels of immunoprecipitated class II MHC complexes. To identify if such polypeptides were derived from the alpha and beta chains, immunoblotting to electrotransferred polypeptides was attempted, with antisera made to synthesized peptides that mimicked eight regions of the alpha and beta chains. These antisera were produced and characterized by dot blotting, ELISA, western blotting, and immunoprecipitation of native and denatured material. One antiserum, to an alpha chain peptide (77-88), blotted to a polypeptide immunoprecipitated by anti-class II MHC antiserum. This observation supported the hypothesis that the alpha and beta chains undergo proteolytic cleavages, possibly in the control of antigen presentation.

It was also demonstrated that I_i and the alpha and beta chains can be phosphorylated under varying culture conditions, but this project was not pursued.

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ABBREVIATIONS

ABTS, 2,2′-azino-di-(3-ethyl-benzthiazoline-sulfonate)

BDT, bis-diazotized tolidine

BSA, bovine serum albumin

ER, endoplasmic reticulum

EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

EDTA, ethylenediaminetetraacetic acid

ELISA, enzyme-linked immunosorbent assay

Endo F, endoglycosidase F

Endo H, endoglycosidase H

FCS, fetal calf serum

HRP, horseradish peroxidase

IEF, isoelectric focusing

IgG, immunoglobulin G

I_i, the electrophoretically invariant glycoprotein which associates noncovalently with class II MHC alpha and beta chains

I_i-CS, chondroitin sulfate form of I_i

Ip, sialic acid-derivatized forms of I_i

KLH, keyhole limpet hemocyanin

LIP, leupeptin-induced proteins

mAb, monoclonal antibody

mIgM, membrane immunoglobulin M

MHC, major histocompatibility complex

NEPHGE, nonequilibrium pH gradient gel electrophoresis

NRS, normal rabbit serum

PBS, phosphate-buffered saline

SDS, sodium dodecylsulfate

TcR, T cell receptor (for class II MHC antigen-presented foreign antigen)

TBS, Tris-buffered saline

TRIS, tris(hydroxymethyl)aminomethane

CHAPTER I

Introduction

A. Specific Aims.

The overall goal of my thesis research has been to understand better the mechanisms that control antigen processing and presentation by class II MHC molecules. Towards this goal I have investigated the following questions:

1. What is the function of I_i? Specifically:

   a. What aspects of the structure of I_i would give a clue to its function and mechanism?

   b. What structural modifications does I_i undergo?

2. Do the class II MHC alpha and beta chains undergo proteolytic cleavage and other structural modifications in the control of antigen processing and presentation?

B. Literature Review.

Antigen processing and presentation in B cells. The presentation of foreign antigen by a B cell to a $CD4^+$ T lymphocyte involves surface immunoglobulin-mediated internalization, as indicated in Fig.

1.B.1. This binding serves to concentrate and focus antigen to B cells which possess immunoglobulin genes rearranged to bind the antigen of interest. This immunoglobulin-mediated internalization limits activation to B cells which have a high probability to produce soluble immunoglobulin specific to the foreign antigen (Rock *et al.*, 1984; Lanzavecchia, 1985; Tony and Parker, 1985).

In many cases antigens have been demonstrated to be altered by the antigen presenting cells, and this alteration can be simulated by limited proteolytic degradation (Shimonkevitz *et al.*, 1983; Buus and Werdelin, 1986a). As is the case with other antigen presenting cells, B cells must process antigen before presentation, (Lanzavecchia, 1985). Although it has been suggested that binding of processed peptide to class II MHC alpha and beta chains could occur on the cell surface (Buus and Werdelin, 1986a), most likely the endosomal compartment where antigen is processed fuses with the class II-containing compartment intracellularly, and binding occurs there. Cresswell (1985) demonstrated that transferrin-neuraminidase conjugates, internalized by means of receptor-mediated endocytosis, can interact with newly synthesized class II MHC proteins and associated molecules, and cause desialylation of I_i and the beta chain. This observation supported the hypothesis that the compartment containing internalized antigen can fuse with the compartment containing the newly synthesized class II MHC alpha and beta chains.

In the compartment containing processed peptide and the class II MHC proteins, certain peptides are selected for binding. Since not all peptides from foreign antigen are presented by the class II complex,

some type of selection criteria probably exists. Attempts to predict which peptides are presented by class II MHC molecules are dealt with in a section below. The process by which class II complexes bind peptide is not understood. When measured with peptides and purified class II MHC antigens, the association rate for binding of class II presented peptides to purified class II MHC antigens (of the respective MHC alleles which restrict presentation of the peptides) has a high affinity at equilibrium (k_d = $3x10^{-6}$ M), but a slow formation rate (k_a = 1 $M^{-1}sec^{-1}$) (Buus *et al.*, 1986b). *In vivo*, one might expect some chemically definable mechanisms to catalyze desetope charging with digested, foreign peptides. Attempts to define mechanisms which might catalyze the interactions of such peptides *in vivo* and/or regulate associations of peptides and class II MHC antigens at the time of charging or discharging, has led to the search for accessory proteins or enzymes which might regulate or catalyze desetope charging.

After the class II MHC alpha and beta chains have bound peptide, the complex is moved to the cell surface. Specific T cells that recognize the class II MHC proteins with bound antigen are activated. The activated T cells release factors which activate the B cell and promote proliferation and differentiation. The B cell matures to a plasma cell and produces antibody of the type that was originally on the cell surface.

Structure of class II-presented peptides. Of the many peptides

obtained by proteolytic digestion of foreign protein antigens, only a few can be presented to T cells. For example, only two peptides of lysozyme (34-45 and 46-61) have been found to account for recognition by 8 of 10 T cell I-A^k-restricted clones which were originally selected with intact antigen (Allen *et al.*, 1985). An additional peptide (81-96) is recognized with I-A^b-restricted clones (Shastri *et al.*, 1985). What primary or secondary structural characteristics define peptides which are finally selected for surface expression in complexes with class II MHC molecules?

Following a structural analysis of the peptides responsible for T cell reactivity to well-studied protein antigens, a hypothesis was developed by DeLisi and Berzofsky (1985), and refined by Margalit *et al.*, (1987). That hypothesis suggested that amphipathicity, the presence of opposing hydrophilic and hydrophobic surfaces, in some nonrandom, organized and stable, secondary structure, is required for a peptide's presentation to the T cell receptor (TcR). They postulated that the hydrophobic surface binds relatively nonspecifically to the desetope of class II MHC antigens, and the hydrophilic surface is recognized by a complementary TcR. This algorithm quantitated the moment of hydrophobicity in 7 amino acid stretches in the periodicities of 100^o (alpha helix), 120^o (3_{10} helix), or 180^o (beta-pleated sheet). It selected peptides with an axial strip of aliphatic, hydrophobic amino acids and, otherwise, variably hydrophilic amino acids and ranked them for best fit. Generally, this technique predicted sequences which stimulated T cell clones, especially when alpha helices were assumed to be present (Spouge *et*

al., 1987).

An alternative method for the prediction of T cell-recognized epitopes has been proposed (Rothbard and Taylor, 1988a; Rothbard *et al.*, 1988b). The authors analyzed known cytotoxic and helper T cell epitopes for similarities and noted similar motifs in the sequences which can be summarized as: (charged or glycine)-(hydrophobic)--(hydrophobic)-(polar or glycine) or, (charged or glycine)--(hydrophobic)-(hydrophobic)-(hydrophobic or proline)-(proline or glycine)-(proline or glycine). This method did not rank putative peptides with regard to their probability or efficiency of class II MHC presentation. The result was the identification of peptides similar to those identified by the DeLisi and Berzofsky method, above.

The chemistry of the class II MHC proteins. The class II MHC molecules in the human are derived from the HLA-D region of the MHC on chromosome 6. Although several loci are present in this region, the expressed class II molecules fall into three isotypes, HLA-DR, HLA-DQ and HLA-DP. Each of these isotype loci contain the genes for at least one expressed alpha and beta chain (Trowsdale and Campbell, 1988).

The alpha chain consists of about 233 amino acids (Figueroa and Klein, 1986) and migrates at about 33-34 kD (Shackelford *et al.*, 1982), as shown in Fig. 1.B.2. The class II MHC alpha chain contains two N-linked oligosaccharide chains, one high mannose chain at Asn_{78}, and a complex N-linked carbohydrate at Asn_{118} (Shackelford and Strominger, 1983; Claesson-Welsh *et al.*, 1986b). Further, there

is evidence that the alpha chain might contain an O-linked carbohydrate chain (Nishikawa *et al.*, 1979; Claesson-Welsh *et al.*, 1986b).

The beta chain consists of about 238 amino acids (Figueroa and Klein, 1986) and migrates at about 27-30 kD (Shackelford *et al.*, 1982). The beta chain contains one N-linked oligosaccharide processed to a complex form and no O-linked chains (Shackelford and Strominger, 1983; Claesson-Welsh *et al.*, 1986b).

It has been shown that both the alpha and beta chains can be sulfated, however that sulfate does not attach to an N-linked carbohydrate (Sant *et al.*, 1988).

The chemistry of the I_i gene products. A nonpolymorphic chain was found in association with the class II MHC alpha and beta chains in both murine (Jones *et al.*, 1978) and human systems (Charron and McDevitt, 1979). This chain has been called the invariant chain, In chain, I chain, I_i, Ii, γ chain, p33 and p32. The I_i gene is on chromosome 5 (Claesson-Welsh *et al.*, 1984), while the MHC is located on chromosome 6. The gene has been cloned and sequenced, and has been shown to have its N-terminus on the cytoplasmic side of the membrane (Claesson *et al.*, 1983b; Kudo *et al.*, 1985; O'Sullivan *et al.*, 1986), as seen in Fig. 1.B.2. The putative transmembrane region is from position 31-56.

The I_i gene has several protein products, utilizing alternate initiation sites and an extra exon. The use of the two in phase AUGs

to produce two different forms of the molecule is depicted in Fig. 1.B.3. The major form, p33, commonly known as I_i, lacks the 16 amino acid region that is included in the alternative forms (p35 or $\gamma 2/\gamma 3$) (Quaranta *et al.*, 1984; Strubin *et al.*, 1986a; Strubin *et al.*, 1986b; O'Sullivan *et al.*, 1987).

The I_i gene also contains an exon (termed 6b, Fig. 1.B.3) which, if included in the transcript, will produce the species called p41 (Yamamoto *et al.*, 1985; Strubin *et al.*, 1986b; O'Sullivan *et al.*, 1987). A species called p43, which is only occasionally seen *in vivo*, is the result of the inclusion of this extra exon and use of the alternate initiation site which adds 16 amino acids (Strubin *et al.*, 1986b; O'Sullivan *et al.*, 1987).

I_i has both two O-linked and two N-linked oligosaccharide units (Machamer and Cresswell, 1982; Charron *et al.*, 1983; Machamer and Cresswell, 1984; Rudd *et al.*, 1985; Claesson-Welsh *et al.*, 1986b). N-linked glycosylation sites are at positions Asn_{114} and Asn_{120}, as predicted from the primary sequence (Claesson *et al.*, 1983b). Both of the N-linked carbohydrate sidechains are processed to a complex form with sialic acid on one or both of these chains (Rudd *et al.*, 1985). One of the O-linked chains might be on Thr_{156}, from experiments presented in this thesis.

A product of the I_i gene is also the core protein of the class II associated chondroitin sulfate proteoglycan (CSPG, I_i-CS), in both mice and humans (Sant *et al.*, 1983; Sant *et al.*, 1984; Sant *et al.*, 1985a; Sant *et al.*, 1985c; Giacoletto *et al.*, 1986). This molecule migrates in a heterogeneous manner from 40 kD to 180 kD

(Giacoletto *et al.*, 1986; Bono *et al.*, 1987; Sorli and Humphreys, unpublished observations). Although a function for this molecule has not been elucidated, it seems that only a small fraction (2-5%) of class II MHC alpha and beta chains contain this species, or that it associates with the class II complex rapidly and transiently (Sant *et al.*, 1985b). Further, I_i-CS seems to be surface-expressed (Sant *et al.*, 1985b) and no class II MHC positive cells have been found that did not also express I_i-CS (Bono *et al.*, 1987).

I_i has been shown to bind palmitic acid at Cys_{28}, located on the cytoplasmic tail, close to the transmembrane region. It is probably added before N-linked glycosylation, since it is present in cells exposed to tunicamycin, which inhibits N-linked glycosylation, and if the addition of the palmitic acid is inhibited with cerulenin, N-linked glycosylation does not occur (Koch and Hämmerling, 1986).

It has also been shown that I_i can be sulfated, however that sulfate does not attach to an N-linked carbohydrate (Sant *et al.*, 1988).

A degradative pathway for a fully processed form of I_i, probably in a post-Golgi compartment, has been described (Blum and Cresswell, 1988; Nguyen *et al.*, 1988). When cells were incubated with leupeptin, two N-terminal remnants of I_i, p21 and p10, were produced. These peptides contain fully processed oligosaccharide chains and were produced maximally 2 to 5 hr after synthesis. The conclusion from these experiments was that these proteins, p21 and p10, were produced from I_i by a leupeptin-insensitive enzyme, and were normally, quickly degraded by a leupeptin-sensitive enzyme to

small undetected peptides.

Associations of I_i with the alpha and beta chains. I_i is cotranslationally associated with the alpha and beta chains via their extracytoplasmic regions (Marks and Cresswell, 1986) and inserted in the endoplasmic reticulum (Claesson and Peterson, 1983a; Kvist *et al.*, 1982). They are transported together through the Golgi, and most or all of the I_i dissociates thereafter (Machamer and Cresswell, 1982; Claesson and Peterson, 1983a). There is a large pool of free I_i in excess of the quantities of the alpha and beta chains (Machamer and Cresswell, 1982; Kvist *et al.*, 1982; Thomas and Humphreys, unpublished observations). Kvist *et al.* (1982) presented evidence that this pool of free I_i never leaves the ER, however observations in this lab imply otherwise. In immunoprecipitates of I_i there are forms of I_i processed in the Golgi (complex N-linked and O-linked carbohydrate) that are in excess of the alpha and beta chains, implying that some free I_i does leave the ER (unpublished observations).

The work of Nowell and Quaranta (1985) supported the idea that fully processed I_i is removed from the class II MHC proteins in an acidic compartment, which might be the same site as that where antigen processing and binding occur. They demonstrated that cells incubated with chloroquine, which disrupts lysosomal function, had class II complexes that contained I_i in association with alpha and beta chains. In untreated cells a far larger proportion of class II MHC

alpha and beta chains lacked I_i. In other experiments, chloroquine has been shown to inhibit antigen presentation *in vitro* in phagocytic and non-phagocytic cells, presumably by inhibiting degradation of antigen (Ziegler and Unanue, 1982; Grey and Chestnut, 1985).

While it has been clearly shown that the class II MHC alpha and beta chains are seen on the cell surface, the question of the surface expression of I_i (either free or in the class II complex) has been less definitive. Koch *et al.* (1982) did an experiment where surface-expressed molecules were labeled with lactoperoxidase-mediated radioiodination, and then immunoprecipitations were attempted. They claimed to be able to immunoprecipitate surface-iodinated murine I_i, without alpha or beta chains, with an anti-I_i antibody; but immunoprecipitated radioiodinated alpha and beta chains without I_i with an anti-class II MHC antibody. They concluded I_i was surface-expressed, but only free of class II complexes. Quaranta *et al.* (1984) could not detect any I_i on the cell surface by immunofluorescence with a monoclonal antibody to I_i. Accolla *et al.* (1985) could not detect I_i on the Raji B cell line, using lactoperoxidase-mediated radioiodination, tritiated $NaBH_4$ to label the oligosaccharide, or flow microfluorometry. Claesson and Peterson (1983a) addressed the question if I_i was present on the cell surface by binding radiolabeled cells to poly(ethylenimine)-coated beads, rupturing the cells, and eluting the bound components. They saw only terminally processed alpha, beta and I_i, indicating that some I_i might be present on the cell surface. Claesson-Welsh *et al.* (1986a)

demonstrated that I_i was present on the surface of immunocompetent Langerhans cells in human skin (1-3% of normal viable epidermal cells) by immunohistochemical staining and indirect immunofluorescence. Elliott *et al.*(1989) could not demonstrate I_i surface expression by radioiodination, biotinylation or immunofluorescence.

It is not entirely clear what are the accessory components of the class II complex and that question is particularly difficult to address since the composition probably changes as the it passes through the cell. Seemingly, no accessory molecules are mandatory members. Kelner and Cresswell (1986) demonstrated that in cells treated with monensin, a carboxylic ionophore which neutralizes acidic vesicles, complexes consisted of alpha/beta/I_i/I_i-CS in a 1:1:1:1 ratio. Presumably this indicated that some of the class II complexes at the point of the Golgi (where monensin has its effect) contained some accessory molecules.

Hypotheses on the function of I_i. It has been hypothesized that I_i is necessary for the assembly, post-translational modifications, and transport of the class II MHC molecules. Several groups have attempted to test this hypothesis with experiments involving the transfer of the I_i and alpha and beta chain genes.

Claesson-Welsh and Peterson (1985) injected the genes for I_i and the alpha and beta chains into *X. laevis* oocytes. They found that when the alpha and beta chains were expressed without I_i, the transport of the class II MHC alpha and beta chains was slowed down

and the two chains appeared incompletely glycosylated. Miller and Germain (1986) did transfection experiments, but only looked at the amount of murine class II (Ia) on the cell surface. They found that there were equal amounts of surface-expressed class II MHC antigens whether the I_i gene was transfected or not. They did not address the kinetics or processing of these molecules. In similar experiments, except in the human system, Sekaly *et al.* (1986) showed that cells transfected with the genes for HLA-DR, DQ and DP antigens could express these molecules on the cell surface in the absence of I_i. When the I_i gene was co-transfected into these cells, the levels of the class II MHC antigens on the cell surface were not increased significantly. These experiments seem to show that I_i was not necessary for the formation of the alpha-beta complex and transport to the cell surface, but might be necessary for the correct processing.

An alternative hypothesis is that I_i might retard the class II MHC alpha and beta chains in an endosomal compartment until it is "completed" with a foreign peptide and released to bulk flow transport to the plasma membrane, in a manner similar to p78 retention of mIgM heavy chain until a light chain is attached and the receptor is fully assembled (Bole *et al.*, 1986). Consequently in cells without I_i, class II complexes would be seen on the cell surface, but incomplete complexes would be present.

I_i might have a more direct role in antigen processing and presentation (Miller and Germain, 1986). Several experiments have been done to address the question whether antigen can be processed and presented in the absence of I_i.

Sekaly *et al.* (1988) transfected fibroblasts with the DR alpha and beta chain genes, without the I_i gene. When these cells were infected with the measles virus, they were able to be lysed by class II-restricted cytotoxic T cells. They concluded that I_i was not necessary for antigen presentation in this case. To address whether I_i was necessary for antigen presentation to T helper cells, Stockinger *et al.* (1989) did an experiment where fibroblasts were transfected with either the genes for alpha and beta chains, or the genes for I_i, alpha and beta. The pair of cells were then assayed for their ability to present native antigen or peptides of the same antigen. It was seen that only the cells with I_i were able to efficiently present native antigen while both types of cells could present pre-processed antigen. This indicated some role of I_i in processing and presentation.

The question as to the function of the various products of the I_i gene is even more difficult to answer. Rosamond *et al.* (1987) showed that when the conversion of I_i to I_i-CS was inhibited by the incubation of cells with a competitive substrate for proteoglycan synthesis (p-nitrophenyl beta-D-xyloside), class II MHC alpha and beta chains were still detectable on the cell surface but the cells were less effective in antigen processing. Sivak *et al.* (1987) performed similar experiments in the murine system, with similar results.

One observation about I_i deserves special mention. Spiro *et al.* (1980) demonstrated that certain subtypes of hairy cell leukemia expressed large amounts of I_i. Narni *et al.* (1986) described that I_i is highly expressed in chronic lymphocytic leukemia. The level is

high enough that they proposed that I_i could be used as a marker for the disease.

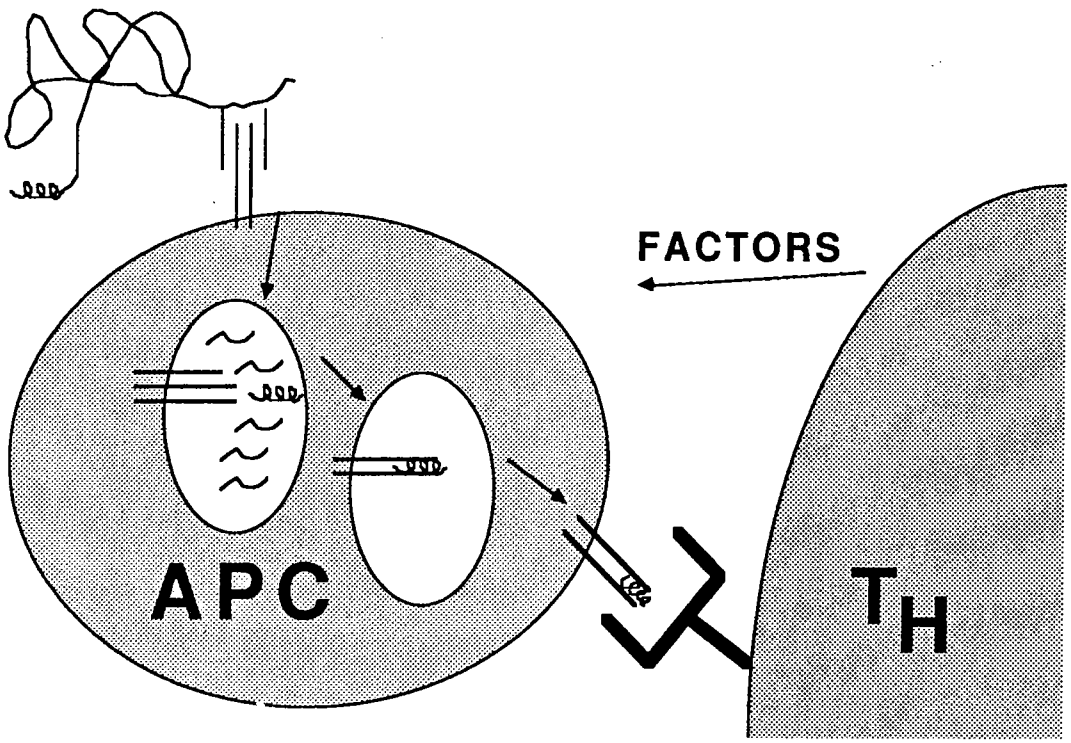


Fig. 1.B.1. Hypothetical scheme of antigen processing and presentation in the B cell. APC, antigen-presenting cell; T_h, T helper cell

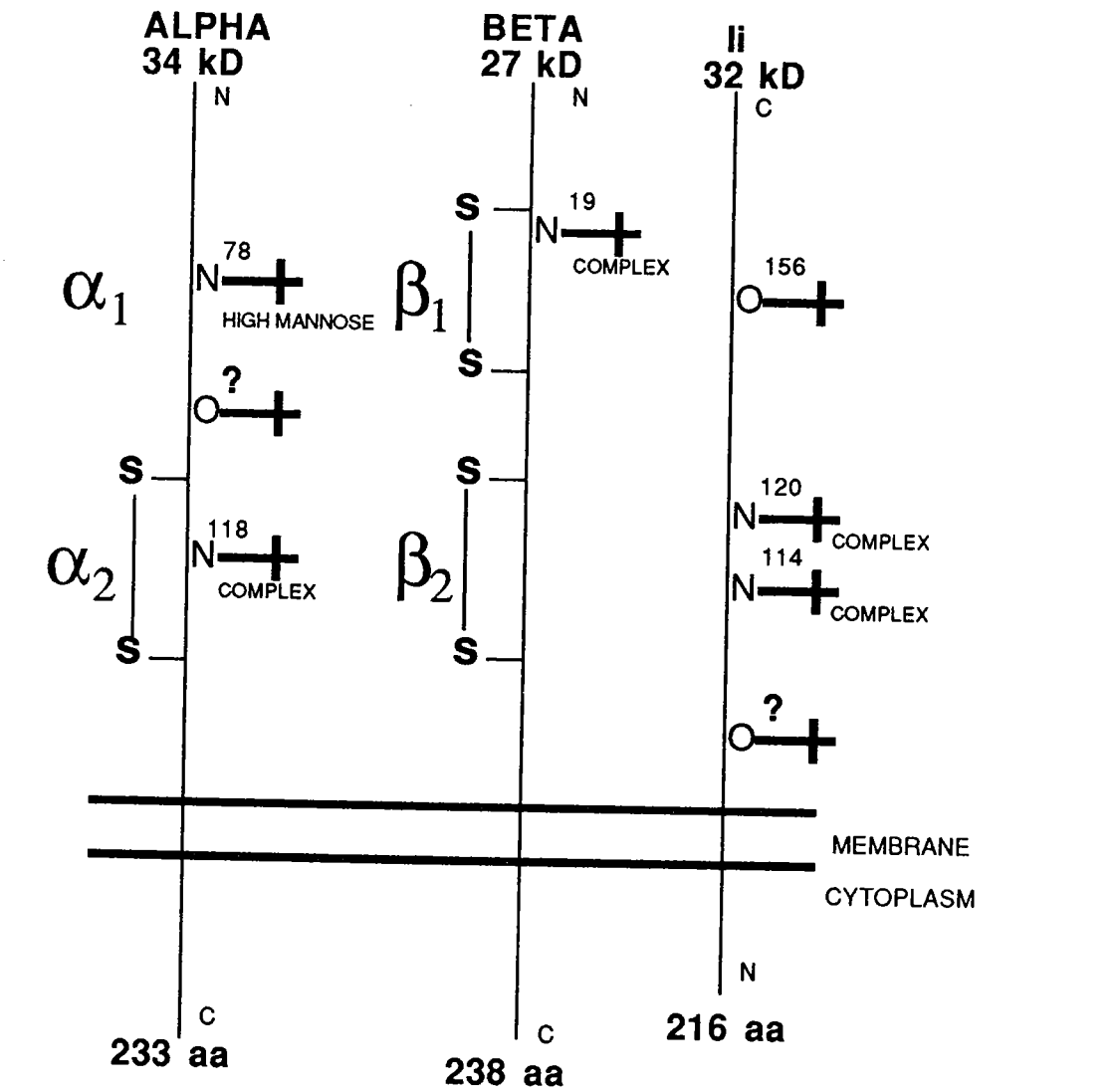


Fig. 1.B.2. Model of the class II MHC alpha and beta chains and I_i. Sites and structures of known and putative glycosylation are indicated.

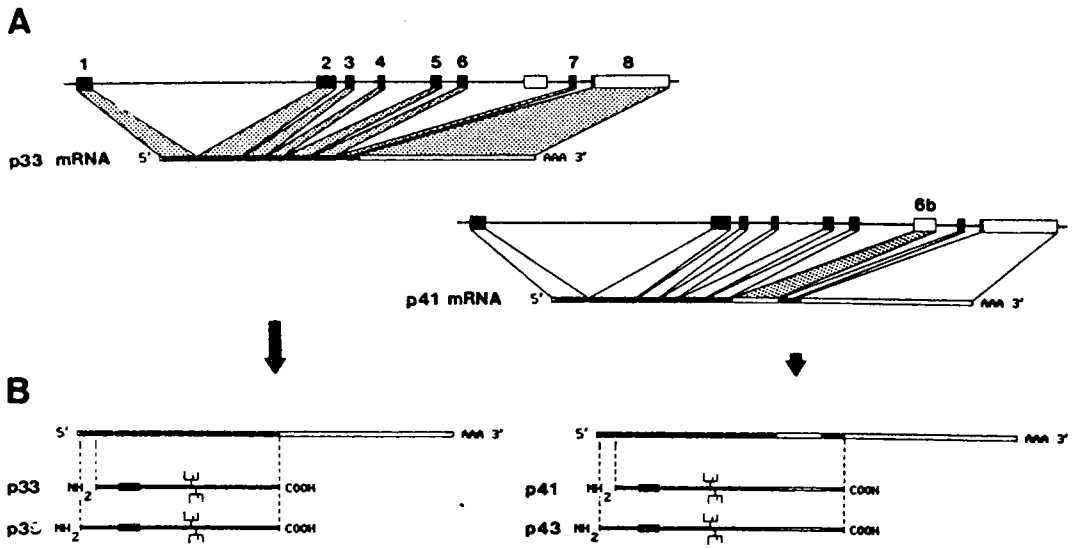


Fig. 1.B.3. Different splicing and initiation of the I_i gene. (A) The structure of the I_i gene, with black boxes as translated regions. In the p41 mRNA exon 6b is translated. Not drawn to scale. (B) Protein products resulting from the use of the exon 6b and the use of alternate initiation sites. I_i is called p33, and $\gamma 2, \gamma 3$ are called p35 in this diagram. Exons are numbered. Most of exon 8 does not appear in the final proteins. (Strubin *et al.*, 1986b).

CHAPTER II

MATERIALS AND METHODS

A. Cells.

Raji was a Burkitt's lymphoma cell line (Pulvertaft, 1965). The Vavy cell line (WSH9023) was purchased from the American Society for Histocompatibility and Immunogenetics Serum Bank and Repository. Its haplotype was HLA-DR 3, DP 1, DQ 2.

Polyclonally *Staphylococcus aureus*-activated B cells were prepared as follows (Nguyen and Humphreys, 1989). Spleens were obtained from immunocompetent patients undergoing splenectomy for surgical or medical indications. Pieces of the spleens were disaggregated by pushing through a fine steel screen, and suspended in RPMI 1640 medium (GIBCO, Grand Island, NY) with 10% fetal calf serum (FCS). A Ficoll-Hypaque gradient (density 1.078) was used to separate the mononuclear cells, which were washed twice and resuspended in the above media with 10% dimethylsulfoxide at a density of 0.4 to 1.0 x 10^8 cells/ml. Cells were quickly frozen and stored in liquid nitrogen. Upon need, splenocytes were quickly thawed and washed twice with the former medium. Approximately 1 x 10^6 cells/ml were activated with a 1/1250 dilution of a 10% solution of formalinized *S. aureus* (Chemicon) in the standard medium. Cells were cultured for 3 days at 37 ^{o}C in 5% CO_2 in air. The cells were then subjected to

the selected protocol below.

Cells were maintained in RPMI 1640 medium supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml), and 10% FCS. Cultures were used in logarithmic growth phase.

B. Anti-Peptide Antisera.

Selection of peptides. Peptides were selected such that antibodies made to them would both immunoprecipitate and western blot to both native and denatured proteins. Towards this goal, a computer program was written in the lab to search for portions of proteins that would likely be antigenic, based on the work of Hopp and Woods (1981, 1983). Mean Kyte-Doolittle hydrophobicity values for sequential sets of 6 amino acids were computed and reported at the 4th amino acid of each set (Kyte and Doolittle, 1982). These regions were then checked for inclusion of prolines, length, and location in the molecule. Portions of proteins that were hydrophilic and met the other criteria, were selected for synthesis. The exception to this logic was the peptide I_i(147-169) which was selected as the putative alpha helical region of I_i that could lie in the class II MHC desetope.

Peptide synthesis. The peptides in Table 2.B.1 were synthesized by the UMMC peptide synthesis facility, with the following exceptions. I needed the peptide I_i(146-169) before an operator was hired to run the facility. Consequently, I synthesized this peptide (on an Applied

Biosystems peptide synthesizer) and uncoupled the peptide myself. This peptide was difficult to obtain in a purified form, so I synthesized it twice. Also, the peptide, I_i(183-193), with addition of a C-terminal tyrosine, was synthesized by Cambridge Research Biochemicals, Ltd., UK.

Syntheses had high coupling efficiencies on each amino acid residue and consequently it was decided that purification was not needed before they were injected into rabbits.

Conjugation of peptide to carrier. The peptides were coupled to keyhole limpet hemocyanin (KLH) with bis-diazotized tolidine (BDT) or with 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), as indicated in Table 2.B.1. For BDT conjugations, 50 mg of the carrier protein was dissolved in 5 ml of a 0.25 M borate buffer, 0.2 M NaCl, pH 9.0. Five mg of the peptide was dissolved in 5 ml of the same buffer and the two protein solutions were mixed. Eight mg of BDT was added to the above mixture and put on ice for 2 hr. The peptide carrier conjugate was dialyzed against PBS for 2 days with frequent solution changes (Bassiri and Utiger, 1972). For EDAC conjugations, 4.8 mg of the peptide in 600 μl H_2O (pH 3.0 adjusted with HCl) was incubated with 2.8 mg of EDAC in 5.4 ml H_2O, pH 3.0 for 15 min at 0° C. A solution of 24 mg of the carrier protein in 0.5 ml of H_2O adjusted to pH 9.0 with $(NH_4)_2CO_3$ was added, and with constant stirring was incubated for 3 hr at 0° C (Tamura *et al.*, 1983). The peptide-carrier conjugate was then dialyzed for 2 days against PBS, with frequent solution changes. The peptide I_i

(183-193) was purchased conjugated to KLH. Peptides were conjugated to bovine serum albumin (BSA), with EDAC, in the same manner as above for assay purposes.

Rabbit injection and bleeding protocol. Rabbits were bled before immunizations began. Peptide conjugates, containing 50 mg of peptide, were injected with complete Freund's adjuvant into New Zealand albino rabbits, boosted with peptide conjugate in Freund's incomplete adjuvant and bled at periodic intervals. Minzhen Xu injected and bleed the rabbits which were immunized with I_i(147-169).

C. Other Antibodies

The monoclonal antibody (mAb) VIC-Y1 was the gift of Dr. W. Knapp (University of Vienna, Austria) (Quaranta *et al.*, 1984). Rabbit antisera N350 and C351, from rabbits injected with the N-terminal peptide I_i(12-28) and the C-terminal peptide I_i(192-211), respectively, were the gifts of Dr. V. Quaranta (Scripps Clinic and Research Foundation) (Quaranta and O'Sullivan, 1986; Giacoletto *et al.*, 1986). Normal mouse ascites was the gift of M. Schmidt (University of Massachusetts Medical School). The rabbit anti-p23,30 serum was prepared to purified class II MHC antigens (Humphreys *et al.*, 1976) and did not react with I_i in solubilized membranes of a class II^-, I_i^+ lymphoblastoid cell line (Spiro *et al.*, 1985).

D. Microsomal Membrane Preparation.

Standard procedures with slight modifications were used (Spiro *et al* 1985; Kessler, 1975). $1x10^8$ cells for cell lines or $5x10^6$ for *S. aureus*-activated B cells were labeled for 3 hr in 5 ml methionine-free or cysteine-free RPMI 1640 medium with 0.5 mCi [^{35}S]methionine (Tran ^{35}S-Label, ICN Radiochemicals, Irvine, CA or New England Nuclear, Boston, MA, Cat. NEG009T or NEG-009L) or [^{35}S]cysteine (New England Nuclear, Boston, MA, Cat. NEG022T), at 37°C in 5% CO_2 in air. Cells were washed with PBS and lysed in 10 mM Tris-HCl buffer, pH 8.1. After removal of nuclear debris by centrifugation, microsomal membranes were prepared by centrifugation at 100,000 x g for 1 hr. Membrane proteins were solubilized in PBS containing 1% Triton X-100. Insoluble material was removed by centrifugation at 100,000 x g for 1 hr.

For the phosphorylation experiments, Raji cells were metabolically labeled for 3 hr with [^{32}P]orthophosphate in phosphate-free RPMI 1640 medium (Irvine Scientific, Irvine CA).

Denatured membranes were prepared as above, except were solubilized in 1.0% Triton, 1.0% SDS, 2.2% 2-mercaptoethanol, 4.3% glycerol, 27 mM Tris. Insoluble material was removed by centrifugation at 100,000 x g for 1 hr, and a tube with the supernatant was put in boiling water for 10 min.

E. Reprecipitation Experiments.

VIC-Y1 mAb-immunoprecipitated complexes were eluted in IEF sample buffer and then were desalted through a Sephadex G-25 column which had been equilibrated with 0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.02% sodium azide, and 0.05% Triton X-100, pH 7.4 (0.05% Triton buffer). Antigens were then subjected to a second immunoprecipitation as described below.

F. Immunoprecipitation.

The detergent-soluble lysates were cleared with formalinized *S. aureus* after addition of normal rabbit serum (NRS). In the case of denatured membranes (section D), 200 μl of solubilized membranes were cleared with the addition of 20 μl NRS and 1.8 ml 50 mM Tris-HCl buffer, 150 μM NaCl, pH 8.1 and subsequent removal of antibody with formalinized *S. aureus*. Separately, 100 μl of 10% protein A-Sepharose (Sigma, St. Louis, MO) in 0.05% Triton buffer was reacted with specific antibody, washed, and then incubated with solubilized lysate. The complexes were washed five times with 0.05% Triton buffer, and the immunoprecipitated material was eluted in IEF sample buffer or SDS sample buffer.

G. Protease Digestions.

A standard immunoprecipitation was performed with VIC-Y1 mAb as described above, but complexes were not eluted from the washed antibody-bead matrix. Instead, enzyme solutions (chymotrypsin and trypsin: 10 mg/ml in 0.05 M Tris-HCl, pH 8.0, 30 ml; or proteinase K: 10 mg/ml in 10 mM Tris-HCl, pH 7.4, 30 ml) were added to the pellets for varying times. The digestions were transferred to tubes with 50 mg urea and 50 μl IEF sample buffer, or 50 μl 2X SDS sample buffer, placed into boiling water for 5 min, snap frozen, and stored at -70^{o}C until the electrophoretic gels were run. Proteinase K, trypsin, and chymotrypsin were purchased from Sigma Chemical Co., St. Louis, MO.

H. Endoglycosidase Digestions.

Endoglycosidase solutions were added to immunoprecipitated protein still bound to the antibody/protein A-Sepharose conjugates in tubes which had been in boiling water for 5 min (Pinter and Honnen, 1988). For endoglycosidase H, this solution was 0.06 units (Genzyme, cat. #ENDO-H-1) in 30 μl of 50 mM sodium phosphate buffer, pH 5.7, with 1.5 mM phenylmethylsulfonyl fluoride (Trimble and Maley, 1984). For endoglycosidase F, this solution was 1.5 units (Boehringer Mannheim, cat. #903-329) in 30 μl buffer (Elder and Alexander, 1985). Control samples received no enzyme. Reaction mixtures were incubated

overnight at 37°C for endo H; at 20°C for 18 hr, then 45 min at 37°C for endo F. Reactions were stopped by boiling, and samples were immediately eluted by incubation with 40 μl of IEF sample buffer and 25 mg urea for 30 min at room temperature.

I. Electrophoresis.

For samples which were subjected to two-dimensional gel analysis, the first dimension was run on a nonequilibrium pH gradient electrophoresis tube gel (NEPHGE); the second dimension was run on a 10% SDS slab gel (Spiro *et al.*, 1985; O'Farrell *et al.*, 1977). For accurate determination of low molecular weight proteins, an 8 M urea, 10% SDS polyacrylamide gel electrophoresis system was used (Swank and Munkres, 1971), in a slab form. Molecular weights were calibrated with standards. Gels were autoradiographed and/or stained.

J. Dot Blots.

Peptide conjugated to BSA was deposited on nitrocellulose membrane using an S&S "Minifold". After the membranes were blocked by incubation in 3% BSA/TBS solution, they were incubated overnight with a dilution of the respective antisera. Following washing, the strips were incubated for 2 hr with [^{125}I]protein A in a 0.5% BSA/TBS solution. The strips were washed with a 0.05% solution of Tween 20 in

TBS, allowed to dry, and exposed to film.

K. ELISA.

Between 0.25 and 8.0 μg of peptide was deposited on the walls of each well of a microtiter plate. After washing, the remaining surface was blocked with BSA. Antisera, diluted to various levels, were incubated in the wells at room temperature for 40 min. After washing, a dilution of horseradish peroxidase coupled goat anti-mouse antibodies was put in each well and incubated at room temperature for 1 hr. After washing again, the substrate solution, 2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonate) (ABTS) was put in the wells and after 10 to 20 min, plates was read in a microtiter plate reader, at 410 nm.

L. WESTERN BLOTTING.

After electrophoresis, by previously described methods, gels and nitrocellulose sheets were equilibrated in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 15 min. After electrophoretic transfer, the nitrocellulose was blocked with a 3% BSA solution, cut into strips, and incubated in an antibody solution overnight. Following 2 washes in TBS with 0.05% Tween 20, the strips were incubated in a solution of [^{125}I]protein A in 0.05% BSA in TBS, for 2 hr. The strips were washed 3 times in TBS with 0.05% Tween 20,

dried, and exposed to film.

Alternatively, for colorimetric detection of adherent antibodies the strips were incubated in a solution of goat anti-rabbit antibodies coupled to horseradish peroxidase, after the initial incubation with the primary antibody. After washing, the strips were immersed in a solution of HRP substrate (4-chloro-1-napthol in diethylene glycol). After adequate color development, the strips were rinsed in water, to stop development.

M. STRIP-OF-HELIX HYDROPHOBICITY INDEX.

For the strip-of-helix hydrophobicity index, the mean Kyte-Doolittle hydrophobic values of 3, 4, 5, or 6 amino acids in an axial strip along one side of a putative alpha helix were calculated from amino acid positions n, n+4, n+7, n+11, n+14, and n+18 in a linear sequence. This computation yielded the mean hydrophobicity of residues in 6 successive turns of a helix lying along a strip of amino acids with alpha carbons within 45^{o} of the indexed residue. When presented graphically, the mean hydrophobicity value of a strip was plotted at position n, the N-terminal amino acid of a strip. Highly amphipathic sequences could be found easily by inspection of the plot and identification of hydrophilic strips one or two residues on either side of the hydrophobic strip. While this strip-of-helix index measured hydrophobicity at the frequency for an alpha helix, algorithms for a beta-pleated sheet (n, n+2, n+4, n+6, n+8, n+10) or

for a 3_{10} helix (n, n+3, n+6, n+9, n+12, n+15) could also be used in connection with visual analysis of the respective graphs.

Sequences of sperm whale myoglobin, hen egg lysozyme, pigeon and beef cytochrome C, hen ovalbumin, pig proinsulin, and foot and mouth disease virus protein VP1 were analyzed with this algorithm, and predictions of antigenic sequences were compared with those of DeLisi and Berzofsky (1985), Spouge *et al.* (1986) and with literature reports as summarized by Spouge *et al.* (1986).

PEPTIDE NAME	SEQUENCE	TYROSINE ADDITION	POSSIBLE ISOTYPE SPECIFICITY	REGION	COUPLER	RABBIT NAME
β1	1-8		DR	N TERM	EDAC	$\beta1^a$
						$\beta1^b$
β2	55-63		DR DP DQ	N TERM	TOLIDINE	$\beta2^a$
						$\beta2^b$
β3	131-138		DR DP DQ	MIDDLE	EDAC	$\beta3^a$
						$\beta3^b$
β4	163-169		DR	MIDDLE	TOLIDINE	$\beta4^a$
						$\beta4^b$
α1	17-25		DR	N TERM	EDAC	$\alpha1^a$
						$\alpha1^b$
α2	39-46	Tyr added C term.	DR DP DQ	N TERM	EDAC	$\alpha2^a$
						$\alpha2^b$
α3	77-88		DR DQ	MIDDLE	EDAC	$\alpha3^a$
						$\alpha3^b$
α4	142-154		DR DP	MIDDLE	EDAC	$\alpha4^a$
						$\alpha4^b$ DIED
Ii	146-169	Tyr sub. for 146		MIDDLE	TOLIDINE	E3
						E4
						E5
						E6
Ii	183-193	Tyr added C term.		C TERM	TOLIDINE	E1
						E2
Ii	194-202			C TERM	TOLIDINE	C10
						C11
					EDAC	L6
Ii	203-211			C TERM	TOLIDINE	C12
						C13
					EDAC	L7

Fig. 2.B.1. Listing of the peptides synthesized and data concerning them.

CHAPTER III

RESULTS

A. The Structural Analysis of I_i Leading to a Hypothesis on the Binding of an Amphipathic, Alpha Helical Sequence in I_i to the Desetope of Class II Antigens and the Hydrophobic Strip-of-helix Algorithm for Selection of T Cell-presented Peptides.

Periodic hydrophobicity in I_i. Given the view that peptides presented to T cells had a propensity to form amphipathic alpha helices, we searched various proteins for such structures. In an analysis of the structure of I_i a distinctive amphipathic alpha helical sequence with a strip of aliphatic hydrophobic amino acids along one side of the helix, and otherwise generally hydrophilic amino acids was found. A hydrophilicity plot of the I_i sequence revealed periodic oscillations in hydrophilicity from Phe_{146} to His_{170} (Fig. 3.A.1, Panel a). An Edmundson wheel projection (Schiffer and Edmundson, 1967) along the axis of the cylinder showed hydrophobicity to be centered in quadrant III (Fig. 3.A.2, Panel b). A sheet presentation of the cylinder's surface showed a distinctive, hydrophobic strip in quadrant III, and the generally hydrophilic rest of the cylinder (Fig. 3.A.2, Panel c). It can be suggested that this amphipathic sequence could associate with class II antigens through

the foreign antigen binding site, until dissociation in the endosomes which have received digested foreign antigen. Thereafter, the I_i sequences, which could otherwise compete for the foreign amphipathic peptides, might polymerize about their hydrophobic strips, perhaps as tetramers, linked also by ionic and hydrophobic bridges on the outsides of the polymers (Fig. 3.A.3).

Prototypic amphipathic peptide. Among a series of protein sequences, the I_i Phe_{146}-His_{170} polypeptide best fit an idealized, long, amphipathic, alpha helix when sequences were analyzed by the program which computed mean hydrophobic indices of amino acids along one side of an alpha helix (strip-of-helix hydrophobicity index; Fig. 3.A.1, Panel b). The index value of I_i Phe_{146} was the greatest found for any strip-of-helix in analyses of cytochrome C, lysozyme, myoglobin, ovalbumin, tetanus toxin fragment C, HLA-DR 3 alpha chain, HLA-DR3 beta chain, HLA-B27, and clotting factor VIII:C (not shown). Consequently this region of I_i could be defined as the "prototypic antigenic amphipathic peptide". Antigenic peptides that most closely approximated this structure would be preferentially presented by class II molecules. Towards the goal of defining such peptides, the strip-of-helix algorithm was produced to compute the hydrophobicity along the side of a helix and to search for peptides that would resemble the structure of I_i(146-170).

Algorithm for hydrophobic strip. This algorithm started from a

consideration of Berzofsky's more general approach to the analysis of frequencies of periodic hydrophobic moments within 7 amino acid stretches (2 turns of an alpha helix) (DeLisi and Berzofsky, 1985; Margalit *et al.*, 1987). However, our specific model was modified to search for peptides which most closely mimicked the "I_i prototypic amphipathic peptide", which was hypothesized to be complementary to the class II MHC desetope which contains a narrow slit hypothetically accepting hydrophobic, preferably aliphatic, residues of digested, foreign proteins.

An algorithm was created to compute the mean hydrophobicity of amino acid residues in a strip extending axially along an alpha helix by averaging Kyte-Doolittle hydrophilicity values of residues at positions n, n+4, n+7, n+11, n+14, and n+18 in the linear sequence of a protein. Such a strip-of-helix hydrophobicity could be calculated over 3 to 6 cycles of the helix, starting with the position n amino acid. In plotting the position of alpha carbons of amino acids in an alpha helix (in which adjacent amino acids are 100^{o} apart), starting with amino acid n, such a strip ran within $\pm 45^{o}$ of the chosen starting point through 5 adjacent turns of the helix before the pattern repeated itself (Fig. 3.A.4). These computations could be altered to identify hydrophobic strips on 3_{10} helices (residues n, n+3, n+6, n+9, n+12, n+15) or in beta-pleated sheets (residues n, n+2, n+4, n+6, n+8, n+10).

Note that in Kyte-Doolittle hydrophilicity plots, the mean hydrophilicity value is for a set of 7 sequential residues plotted at the 4th amino acid position in the series, but in the strip-of-helix

plot, the mean hydrophobicity value is for amino acids along a +45° strip from 3 to 6 turns of a helix in the C-terminal direction from the index point (Kyte and Doolittle, 1982).

Strictly speaking, this index measured periodic hydrophobicity and not amphipathicity as defined by Berzofsky, for it evaluated only hydrophobic amino acids in a strip along the side of a hypothetical alpha helix, and ignored contributions to a moment of hydrophobicity from hydrophilic or hydrophobic side chains elsewhere on the cylinder (DeLisi and Berzofsky, 1985).

From the highest scoring hydrophobic strips-of-helix, we identified those with flanking hydrophilicity by finding at least one adjacent strip (within ±2 amino acids from the hydrophobic strip) which had a mean hydrophobicity score of -1 or less. Graphically, the highly hydrophobic index strip was frequently bracketed by multiple, variably hydrophilic strips (Fig. 3.A.5). In contrast, transmembranal regions of a protein had a series of hydrophobic strips without any adjacent hydrophilic strips, reflecting the generally hydrophobic character of all sides of the helical forms of transmembranal peptides. We then excluded from the 5 highest scoring peptides, those which contained a proline which could break the alpha helical pattern. For consistency in ranking analyses (in contrast to selections for syntheses, see below), the boundaries of peptides were defined to be the first and last amino acid in the hydrophobic strip.

Our computerized protocol then consisted of: (1) calculating strip-of-helix hydrophobicity values for 3, 4, 5 and 6 turns of a putative helix (8, 12, 15 and 19 amino acids in length), (2) printing

of sequence position, amino acid and index value in tabular form, and index value and sequence position graphically, (3) listing of the 5 most highly ranked peptides, (4) indicating those which did not have at least one adjacent strip (±1 or 2 amino acids from position n with hydrophobicity score -1.0 or less, (5) indicating those peptides which contained a proline and (6) indicating peptides which contained a lysine +1 or +3 positions after the C-terminal hydrophobic residue.

The design of this algorithm was based upon several observations. For any protein only the five most hydrophobic strip-of-helix peptides were examined because previously studied proteins had a maximum of four antigenic sites (in hen egg lysozyme). Also, one might suspect that competitive binding of peptides to class II antigens would generate a hierarchical order in presentation and thus scoring for 3 or 4 peptides would be sufficient, having allowed for the detection of one transmembrane peptide. In this study, no experimentally antigenic peptide which possessed a hydrophobic strip-of-helix was found to be excluded from the five most highly scoring strips. Selection of -1.0 as the "adjacent hydrophilicity" threshold was based on an examination of sperm whale myoglobin, pigeon cytochrome C, hen egg lysozyme, and hen ovalbumin. This examination showed that all experimentally antigenic peptides had at least one adjacent hydrophilic strip of index -1.0 or less. In addition, few peptides that were not reported to be experimentally antigenic were included at this threshold value. Finally, since the length of experimentally antigenic peptides in the proteins studied varied from 11 to 17 amino acids, analysis of 4 to 6 helical turns (11 to 19 amino acids) was appropriate.

Analysis of classical protein immunogens. Amino acid sequences of sperm whale myoglobin, hen ovalbumin, hen egg lysozyme, beef cytochrome C, pigeon cytochrome C, and foot and mouth disease virus protein VP1 were searched for their highest scoring strip-of-helix hydrophobicity indices at 3, 4, 5 or 6 turns of a helix. The 5 highest scoring peptides in each protein were then tested for an adjacent strip with a hydrophobicity index of -1.0 or less and for the absence of prolines in the hypothesized amphipathic helical region. Strip-of-helix hydrophobicity plots at 5 turns analysis of the highest ranked peptides in sperm whale myoglobin, hen ovalbumin, and beef cytochrome C are presented in Fig. 3.A.5. The peptides selected according to our protocol are listed in Table 3.A.6. We found a close concordance among the peptides chosen with our algorithm, those selected with the DeLisi and Berzofsky procedure, and those found experimentally to be antigenic. The DeLisi and Berzofsky procedure and our selection procedure both included about the same number of "extra" amino acids than those found in experimentally antigenic peptides. The proteases used to obtain experimentally antigenic fragments probably did not cleave out exactly the epitopes needed for binding to the class II desetope. The DeLisi and Berzofsky method and this approach each failed to identify two peptides which experimentally had been found to be T cell-presented. Overall, however, the strip-of-helix hydrophobicity algorithm and the DeLisi and Berzofsky procedure generally predicted the principal peptides which were T cell-presented.

In order to visualize a helical peptide an Edmundson wheel (Fig.

3.A.2, Panel b) can be used to present amino acid residues along the axis of an alpha helix with successive residues 100° apart (Schiffer and Edmundson, 1967). This is the simplest approach to judge clustering of hydrophobic residues about one side of a helix, but it is limited because the axial orientation of residues along one side of the helix is not easily visualized, and residues begin overlapping positions in the wheel after 18 amino acids. A sheet projection of the helix (cut at one position in the Edmundson wheel) displays the linear array of amino acids down each of four quadrants along the axis of the helix. This projection was useful in looking for potential interactions between groups in hydrophilic quadrants II and IV of adjacent helices, given a hypothesis that a polymer was formed around hydrophobic quadrant III associations.

Function of the prototypic amphipathic region. Seeing the "prototypic T cell-presented peptide" within I_i, led to the view that this amphipathic helix might bind to the class II MHC desetope. A diagrammatic representation of the putative structure is shown in Fig. 3.A.7. One could speculate that the function of I_i was, in part, to keep that class II MHC site free of endogenous peptides until reaching endosomes where foreign antigen was digested by proteases. The dissociation of I_i from the class II complex could be promoted by several means, including acidic pH or proteolytic cleavage, but the released I_i amphipathic helix, in either small or large fragments of I_i, might compete with foreign peptides for binding to the class II

desetope. However, analysis of the I_i helical peptide's structure indicated that the peptide probably could no longer bind to the class II MHC alpha and beta chains because it would self-associate tightly through: (1) a core of interdigitating, aliphatic side chains, (2) ionic bridges at the external surface of the polymer, and (3) hydrophobic interactions near the C-terminus of the helices. These interactions were demonstrated clearly in scaled, molecular models of two helices (Fig. 3.A.3). While formation of dimers, trimers, pentamers or other aggregates could not be ruled out, association of these I_i peptides as a tetramer would appear to be the sterically favored polymer. In nature, kinetics of release and dimerization of I_i structures might govern what polymeric form would prevail.

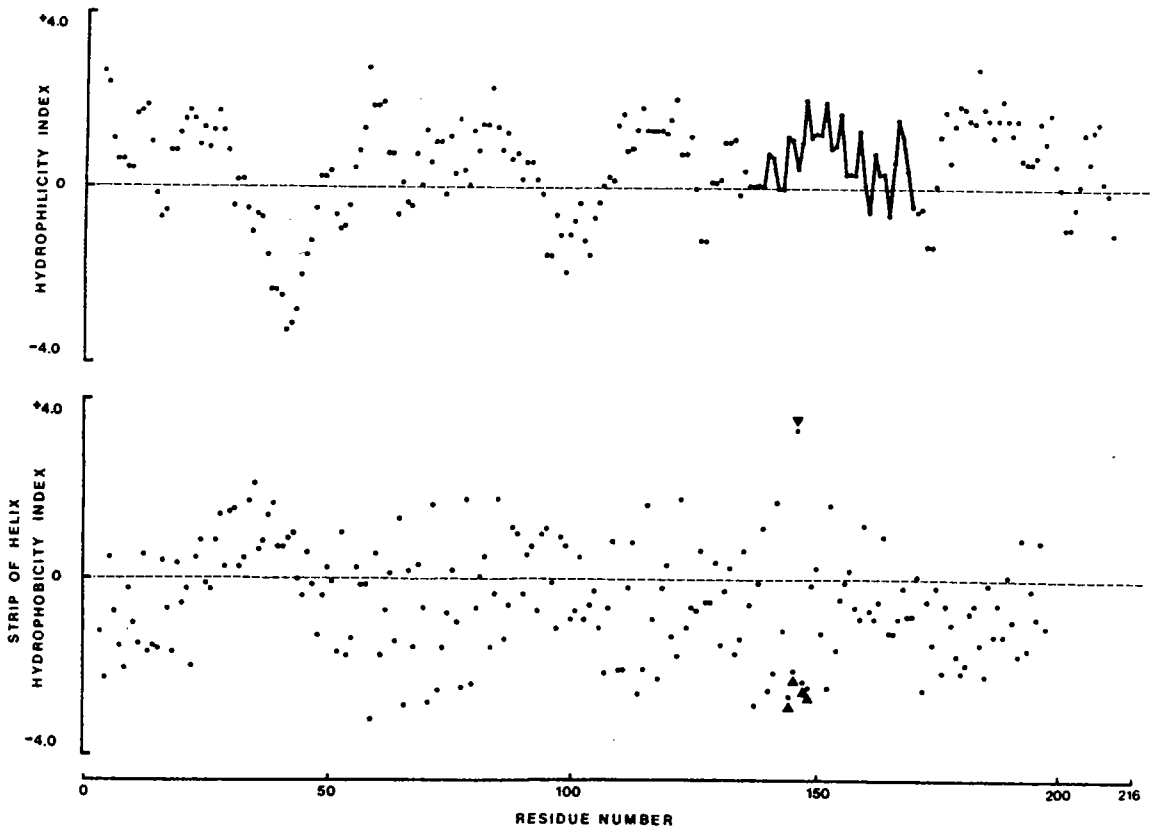


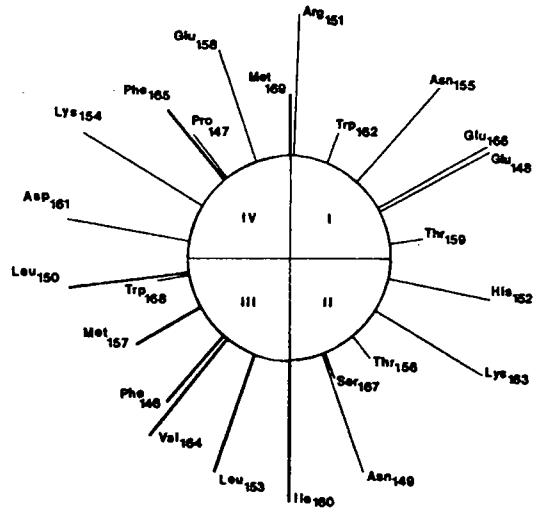
Fig. 3.A.1. Hydrophobicity (Kyte-Doolittle values) and periodic hydrophobicity (in an alpha helix) plots of the I_i sequence. (A) Mean Kyte-Doolittle hydrophobicity values of 6 sequential amino acids from the position indicated in the abscissa were plotted. (B) Mean Kyte-Doolittle hydrophobicity values of 6 amino acid side chains in a strip along a putative alpha helix, *i.e.*, positions: n, n+4, n+7, n+11, n+14, n+18 were plotted. Arrowheads indicate the very hydrophobic strip beginning at Phe_{146} and the four adjacent hydrophilic strips beginning at positions 144,145 and 147,148.

ii PEPTIDE: 146 – 175

AMINO ACID SEQUENCE

146 150 155
Phe - Pro - Glu - Asn - Leu - Arg - His - Leu - Lys - Asn -
160 165
Thr - Met - Glu - Thr - Ile - Asp - Trp - Lys - Val - Phe -
170 175
Glu - Ser - Trp - Met - His - His - Trp - Leu - Leu - Phe

EDMUNDSON WHEEL PROJECTION



SHEET PROJECTION

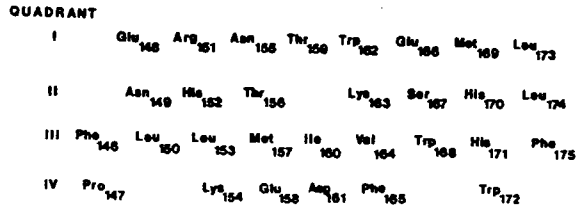


Fig. 3.A.2. Peptide sequence presented in an Edmundson wheel and in a sheet projection of the cylinder's surface. (A) Amino acid sequence. The amino acid sequence between residues Phe_{146} and Phe_{175} is given. (B) Edmundson wheel. This projection placed each residue at increments of 100^{o} about a circle. Hydrophobic values were presented as thick lines from the circumference, and hydrophilic values as thin lines; the lengths were proportional to the absolute Kyte-Doolittle values for each amino acid. Quadrants were arbitrarily assigned. Most of the hydrophobic values fell in quadrant III. (C) Sheet projection. The amino acid placements about the cylinder represented in the Edmundson wheel pattern were displayed as a sheet (cutting the cylinder along the boundary between quadrants I and IV). The hydrophobic strip is seen in quadrant III.

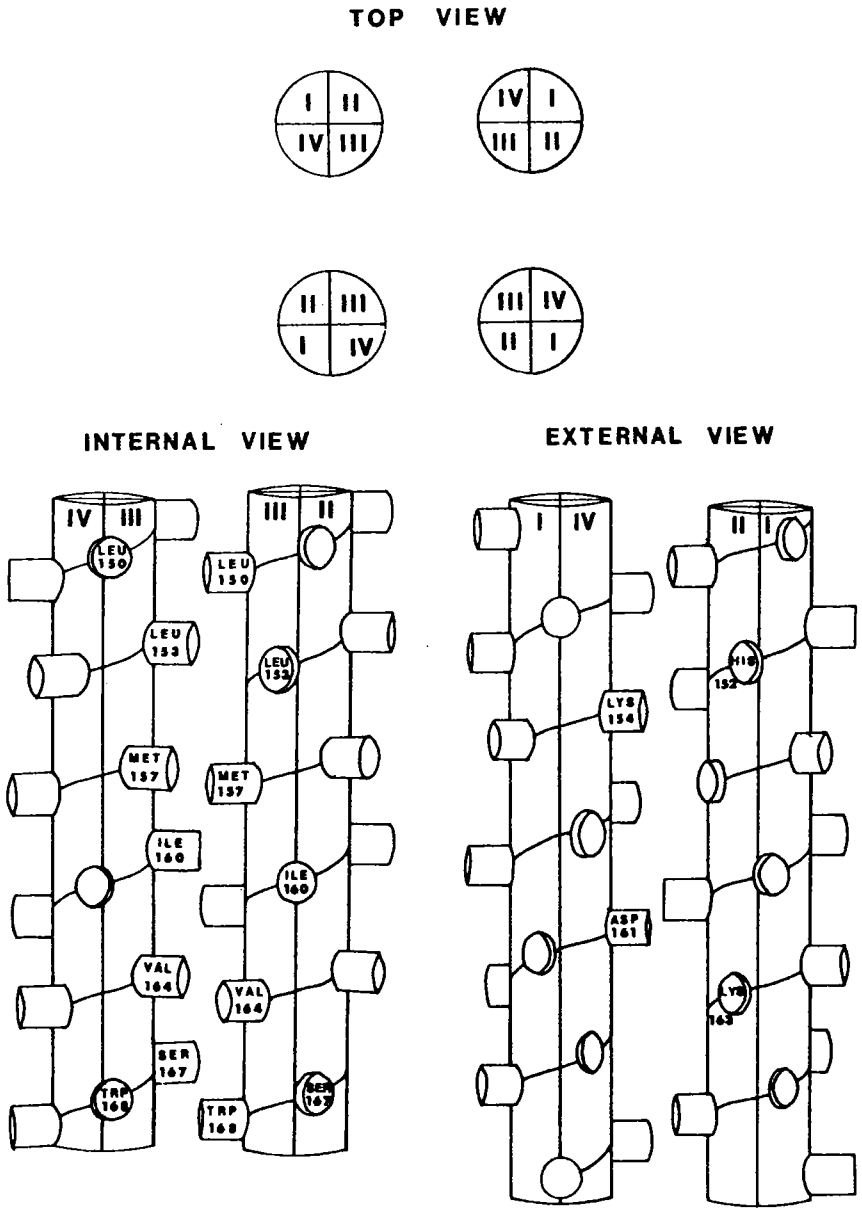


Fig. 3.A.3. Polymerization of the I_i peptide, three views. In the top view, quadrant orientation of peptides aligned with N-termini together in a putative tetramer follows from the association of hydrophobic residues of quadrant III. In the internal view, hydrophobic residues Leu_{150}, Met_{157}, and Val_{164} interdigitated between residues Phe_{146}, Leu_{153}, Ile_{160}, and Ser_{167}, respectively, in a zipper-like fashion which was then repeated clockwise around the tetramer. In the external view, ionic bridges between His_{152}-Lys_{154} and Lys_{163}-Asp_{161} are possible, the His_{152}-Lys_{154} pair being pH sensitive.

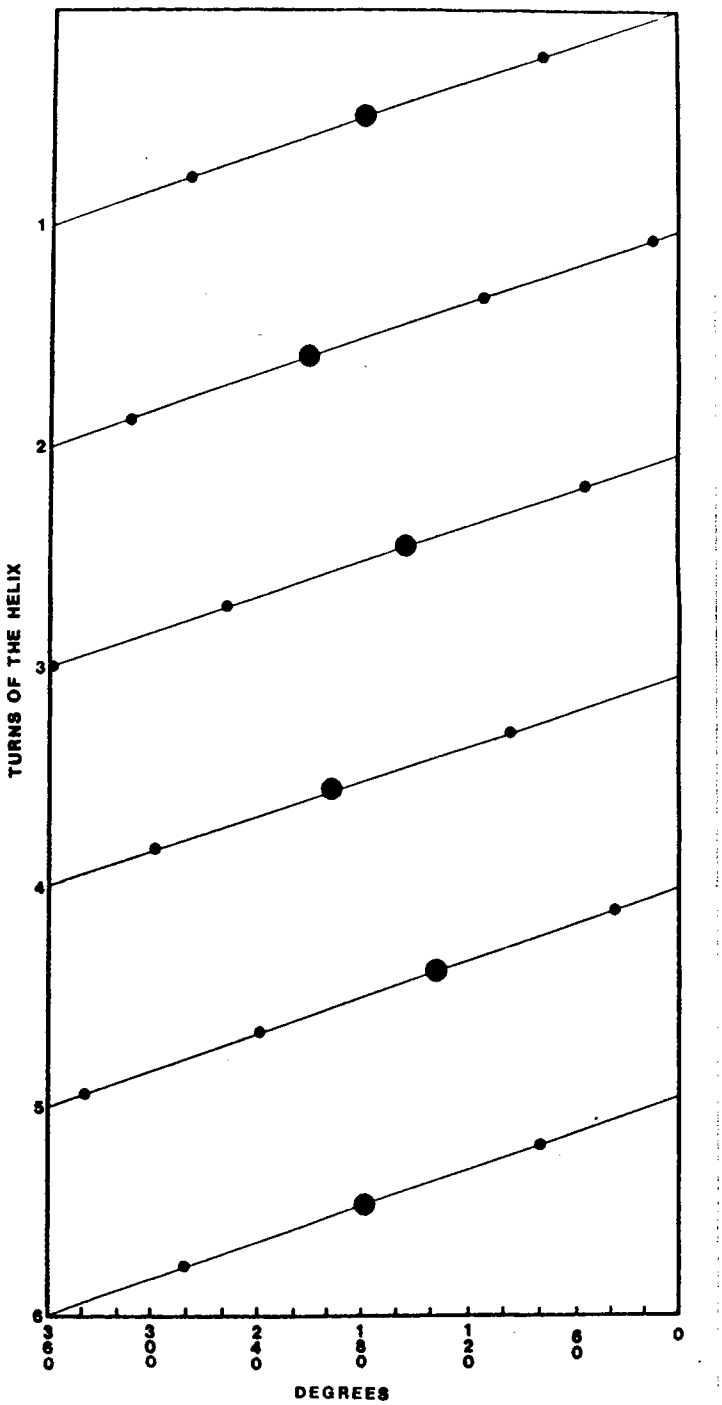


Fig. 3.A.4. Sheet projection of an alpha helix. The positions of alpha carbons in an alpha helix, 100° from each other, are plotted. The heavier dots are the residues within 45° from the first residue in a strip which is centered at 180°. Each amino acid in the strip is a "turn", in the sense that floors are reached in a spiral staircase. Three turns are thus found in two full cycles of the helix.

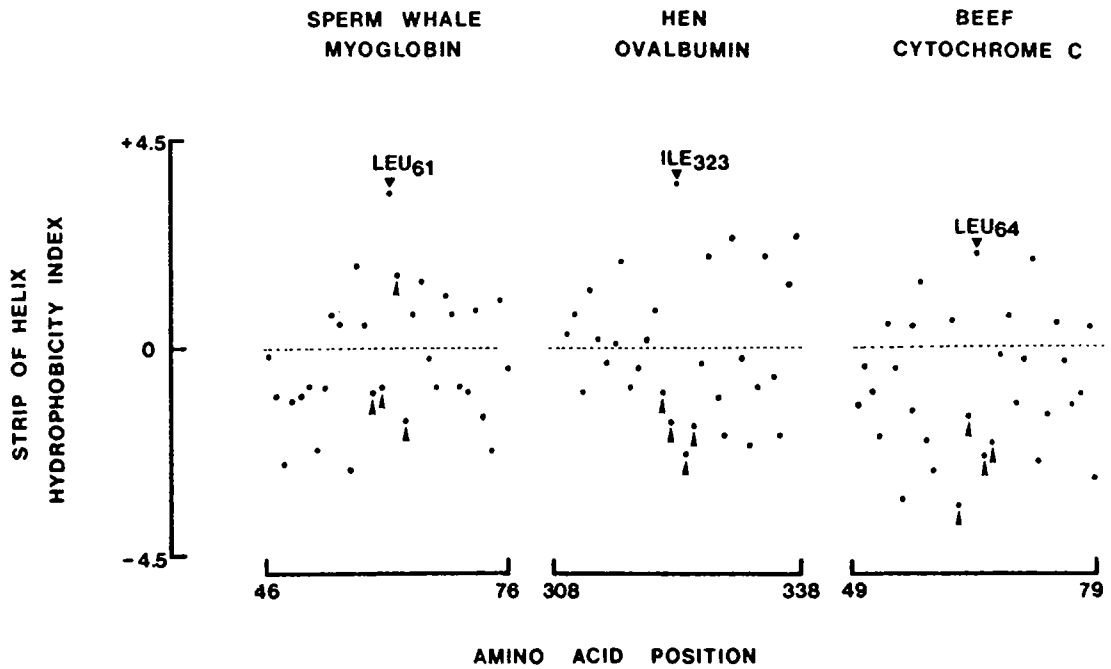


Fig. 3.A.5. Representative program-selected peptides. Strip-of-helix hydrophobicity values for regions containing highest-ranked peptides are shown for amphipathic regions from sperm whale myoglobin, hen ovalbumin, and bovine cytochrome C. The singly highest-ranked peptide is indicated with a triangle and the amino acid position. The values of the four adjacent, relatively hydrophilic strips (2 on the N-terminal side, 2 on the C-terminal side) are indicated with tapered arrowheads.

Table 3.A.6. Proposed T Cell-Stimulating Amphipathic Peptides.

Predictions of this algorithm		Reported Antigenic Peptides
PEPTIDE	INDEX*	
Sperm whale myoglobin		
Leu_{61} - Ile_{75}	3.18	68-78
Pro_{100} - Val_{114}	3.08	102-118
Phe_{123} - Leu_{137}	2.40	132-146
Val_{21} - Ser_{35}	2.26	none
Hen egg Lysozyme		
Ile_{88} - Gly_{102}	2.86	81-96
Leu_{84} - Ile_{98}	2.76	81-96
Gly_{71} - Ser_{85}	1.78	74-86
Ser_{81} - Ala_{95}	1.78	81-96
None found		46-61, 108-119
Hen Ovalbumin		
Ile_{323} - Ala_{337}	3.36	323-339
Pigeon cytochrome C		
Leu_{94} - Lys_{104}	3.13	94-104
Ile_{85} - Ala_{96}	2.70	none
Phe_{10} - Glu_{21}	2.60	none
Thr_{78} - Ala_{89}	2.20	none
Pig proinsulin		
A Ile_{2} - A Leu_{16}	2.76	A 4-A 14
A Leu_{16} - B Ser_{9}	2.70	B 5-B 16
A Ser_{9} - B Val_{2}	2.70	A 4-A 14
B Leu_{11} -B Phe_{25}	2.02	B 5-B 16
A Cys_{6} - A Cys_{20}	1.96	A 4-A 14
Beef cytochrome C		
Val_{3} - Cys_{17}	1.62	11-25
Leu_{32} - Phe_{46}	1.46	none
Ile_{57} - Pro_{71}	1.40	66-80
Foot and mouth virus VP1		
Leu_{144} - Thr_{158}	2.98	141-160
Val_{62} - Leu_{76}	2.76	none
Leu_{148} - Ser_{162}	2.56	141-160
Gln_{28} - Val_{42}	2.44	none

*Strip-of-helix hydrophobicity index at 5 turns is given except for pigeon cytochrome C which is presented at 3 turns because the highest ranking antigenic peptide Leu_{94} - Lys_{104} is at the C-terminus. Peptides which contained prolines or failed to meet threshold criteria for adjacent hydrophilic strips, are not listed.

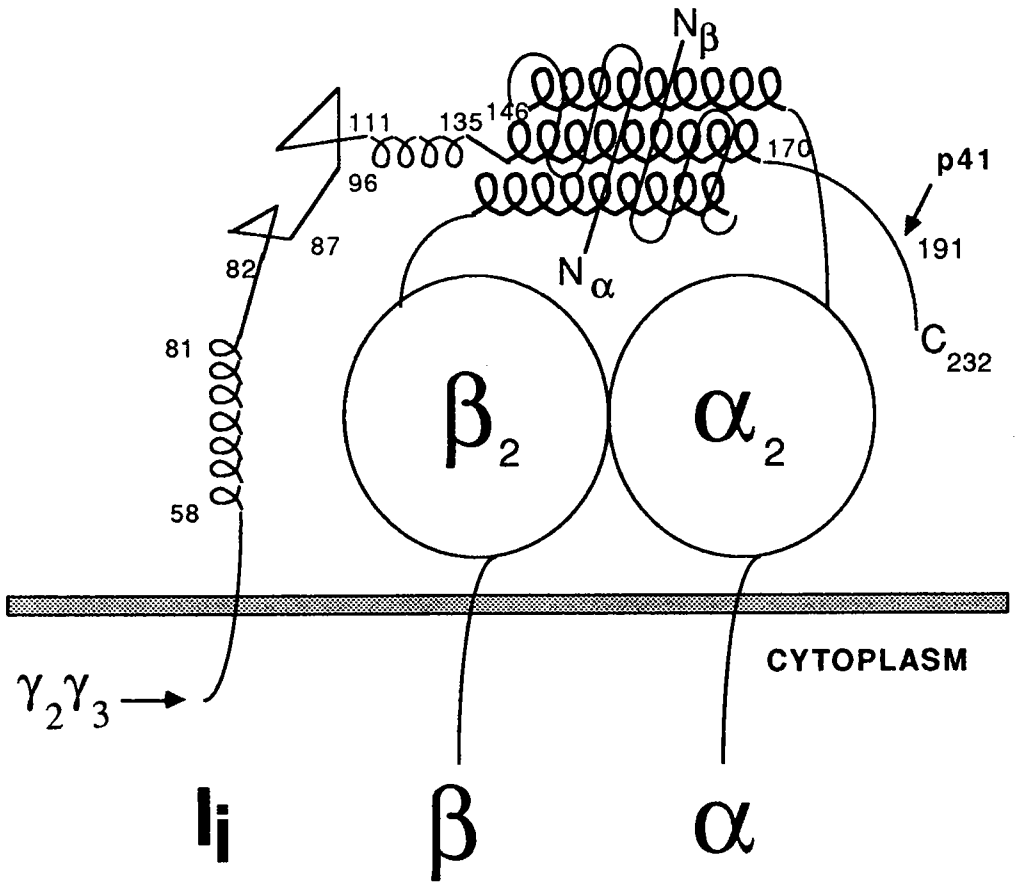


Fig. 3.A.7. Hypothetical model of I_i and the alpha and beta chains. I_i is shown resting in the desetope of the class II MHC proteins, prior to the binding of foreign antigen. The site of the addition of the extra exon to generate p41 and the site of the addition of 16 amino acids at N-terminus to generate γ2 and γ3 are shown. Sites of certain structural features also are indicated. Additional area of interaction between I_i and the alpha and beta chains also probably exist.

B. Proteolytic Cleavage of I_i to p25.

Introduction. To pursue the working hypotheses on the function of I_i, we have examined structural changes of the molecule. On two dimensional gels of immunoprecipitates of I_i and class II molecules a protein called p25 has been observed (Fig. 3.B.1). In this study we have shown that p25 is the C-terminal portion of the I_i molecule, containing the amphipathic alpha helix (146-170). Since p25 is seen when immunoprecipitates are done in the presence of protease inhibitors, and maximal labeling is seen at 20-40 min in pulse-chase experiments, we feel it is not an artifact of the protocol. Cleavage of I_i to p25 appeared not to be determined by a specific substrate sequence, but by a domain character in I_i, since three proteases with varying specificities preferentially cleaved about the immediate exomembrane region of I_i. Further, all p25 molecules were sensitive to treatment with endoglycosidases F and H and, therefore, must be high mannose-derived and, thus, generated in the endoplasmic reticulum or cis-Golgi apparatus.

Re-immunoprecipitations of denatured, I_i-associated proteins. Detergent-solubilized, [^{35}S]methionine-labeled, microsomal membranes were subjected to immunoprecipitation with VIC-Y1 mAb, yielding I_i and associated molecules (Fig. 3.B.2, Panel A). These immunoprecipitated antigens were denatured with 9 M urea, desalted by

passage through a Sephadex G25 column equilibrated with 0.05% Triton buffer and immunoprecipitated a second time with various antisera. Two rabbit antisera to peptides in the carboxy terminal portion of I_i, anti-I_i(183-193) (E1) and anti-I_i(192-211) (C351), immunoprecipitated p25 and I_i (Fig. 3.B.2, Panels E and C, respectively).Rabbit antiserum to a N-terminal peptide (12-28) of I_i (N350) failed to immunoprecipitate p25 but recognized I_i (Fig. 3.B.2, Panel D). A second immunoprecipitation with VIC-Y1 mAb also recognized some of the denatured forms of I_i, but not p25. VIC-Y1 is a monoclonal antibody and may be to a more native structure. Consequently it is difficult to conclude anything from this particular reprecipitation. An antiserum to irrelevant proteins (E4) failed to immunoprecipitate I_i or any I_i-related proteins.

Absence of Cys_{28} from p25. Since only one cysteine was present in I_i, at sequence position 28, [^{35}S]cysteine-labeled proteins were immunoprecipitated and compared to [^{35}S]methionine-labeled and immunoprecipitated proteins (Fig. 3.B.3). While [^{35}S]-labeled I_i was present in both methionine and cysteine radiolabeled samples, [^{35}S]-labeled p25 was found in methionine- and not in cysteine-radiolabeled cells. Control precipitates with NRS were blank (results not illustrated). The cleavage site to form p25 was judged to occur after Cys_{28} in the primary sequence of I_i.

N-terminal sequence of p25. In order to confirm that the molecule known as p25 was the C-terminal portion of I_i, we intended to do an N-terminal sequence of p25 (detecting [^{3}H]leucine residues) and compare it to the known sequence of I_i. The three major obstacles to overcome were (1) purification of p25 from other membrane proteins including class II MHC molecules and I_i, (2) production of enough p25 from which to get a sequence and (3) production of p25 with enough [^{3}H]leucine incorporated to allow detection of leucine residues. The experimental strategy was to label Raji cells with [^{3}H]leucine and purify I_i and p25 on an immunoaffinity column with a covalent linkage between the antibody anti-I_i(183-193) and the support. This would eliminate most of the cold protein from the final immunoprecipitate to enable more radioactive protein to be loaded on a gel, which would be sliced to isolate p25. Unfortunately, the incorporation of leucine into p25 was low. This would make the detection of leucine residues difficult, since the signal would not be much over the noise. Consequently, the goal of matching the sequence of leucine residues to the I_i sequence seemed difficult to attain, and this experiment was abandoned.

Maximal Production of p25 20-40 min after synthesis. Polyclonally *S. aureus*-activated B cells were labeled with [^{35}S]methionine for 10 min, then chased for 0, 10, 20 and 40 min. Microsomal membranes were immunoprecipitated with anti-I_i(183-193) and separated by 2 dimensional gel electrophoresis (Fig. 3.B.4). p25 appeared maximally

between 20-40 min following synthesis.

Proteolytic digestions of native I_i. In order to analyze whether the *in vivo* cleavage of I_i to p25 reflected great specificity of a protease for a particular I_i sequence, or the effect of a secondary, structural (domain) restriction (for example, as found about the hinge region of IgG; Porter, 1959), the susceptibility of native I_i to several proteases with varying substrate sequence specificities was tested (Fig. 3.B.5). Trypsin (EC 3.4.21.4) cleaved at the carboxy side of lysyl or arginyl residues. Chymotrypsin (EC 3.4.21.1) and Proteinase K (EC 3.4.21.14) cleaved at the carboxy side of aromatic residues and sometimes after hydrophobic residues. I_i and associated molecules, still bound to the antibody VIC-Y1-protein A Sepharose beads, were subjected to digestion with these enzymes (Fig. 3.B.5). In each instance, I_i was digested to a series of proteins about 25 kD, some of which overlapped naturally occurring p25. The 25 kD proteins were relatively resistant to further digestion, but at longer digestion times were reduced to smaller peptides.

Glycosylation of p25. Anti-class II MHC serum immunoprecipitates of [^{35}S]methionine-labeled proteins which were subjected to endoglycosidase F treatment demonstrated a decrease in molecular weight of p25 from 25 to about 10.5 kD (arrows, Fig. 3.B.6). A third

gel of mixed, equal quantities of the control and endoglycosidase F-treated samples confirmed the decrease in weight of p25 and of I_i and N-linked Ip forms. The identities of alpha and beta chains of class II MHC antigens, of a nonglycosylated I_i precursor (I_i), of four I_i forms with N-linked sugars (IpN) and five I_i forms with O-linked carbohydrate side chains (IpO), as presented in Fig. 3.B.1, were established previously (Machamer and Cresswell, 1984). The weight of the 10.5 kD product was confirmed by electrophoresis in an urea/SDS polyacrylamide gel (not illustrated).

In order to establish whether the N-linked sidechains were composed of high mannose or complex forms, similar immunoprecipitates with anti-I_i(183-193) serum, with VIC-Y1 mAb, and with anti-class II MHC serum, were digested with endoglycosidase H (Fig. 3.B.7). In each instance, p25 was reduced in weight to 10.5 kD. This fact was confirmed in gels of mixed, equal samples of control and endoglycosidase H-treated samples (not illustrated).

Since all p25 was subject to digestion with endoglycosidase H, it was concluded that the N-linked carbohydrate chains on the I_i pool degraded to p25 were all high mannose forms. One I_i form (the most basic of the IpN spots) was not fully subject to digestion with endoglycosidase H but was digested with endoglycosidase F. It therefore represented molecules in which carbohydrates were probably converted to complex forms. Furthermore, while VIC-Y1 recognized Ip-O-linked forms, anti-I_i(183-193) did not. Since p25 was recognized with anti-I_i(183-193), it was concluded that it did not have the O-linked carbohydrate forms of Ip-O(1-5). p25, therefore, was

derived from relatively "early" forms of I_i with high mannose sidechains and without O-linked sugars.

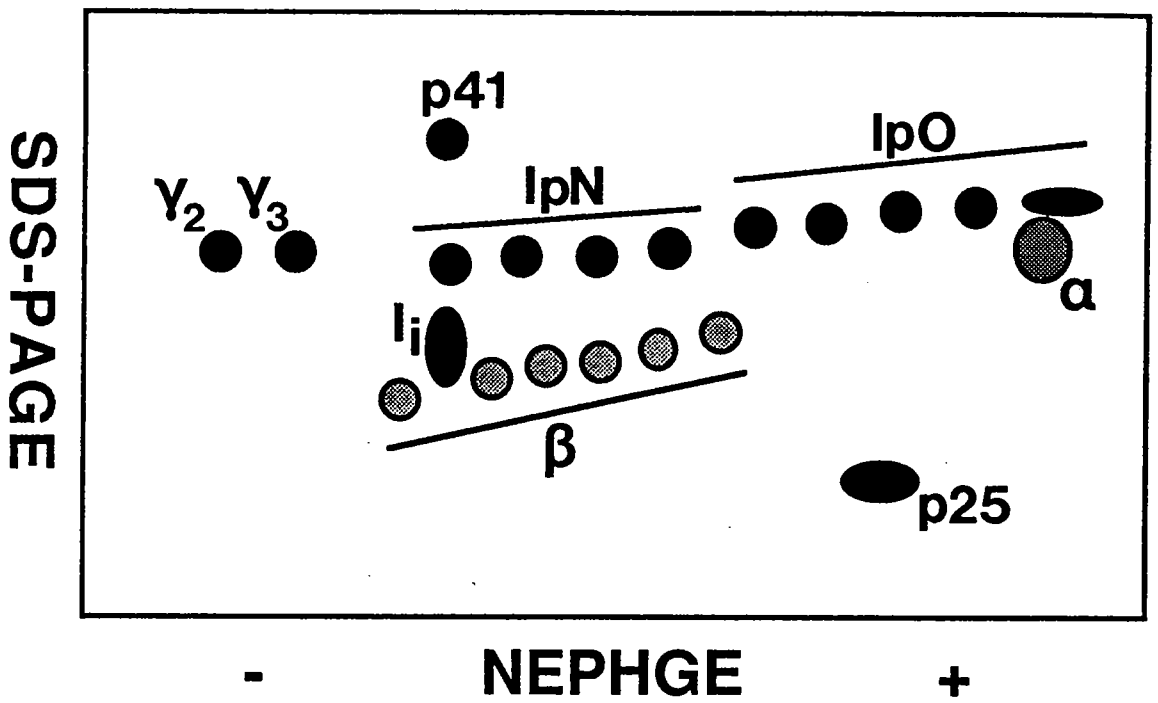


Fig. 3.B.1. Diagram of the distribution in 2-dimensional (NEPHGE/SDS) gels of class II MHC and associated proteins. Alpha and beta are two polymorphic chains coded by the class II genes of the MHC. I_i is the electrophoretically invariant chain noncovalently associated with the alpha and beta chains. IpN is a series of I_i molecules which have N-linked glycosylations. IpO is a series of I_i molecules which have additional O-linked glycosylations. γ2 and γ3 are I_i forms translated from an upstream alternate start site of the I_i structural gene, resulting in 16 extra amino acids. p41 is an I_i form transcribed with an extra exon coding for an extra 64 amino acids after Lys_{192}. p25 is a cleavage product of I_i.

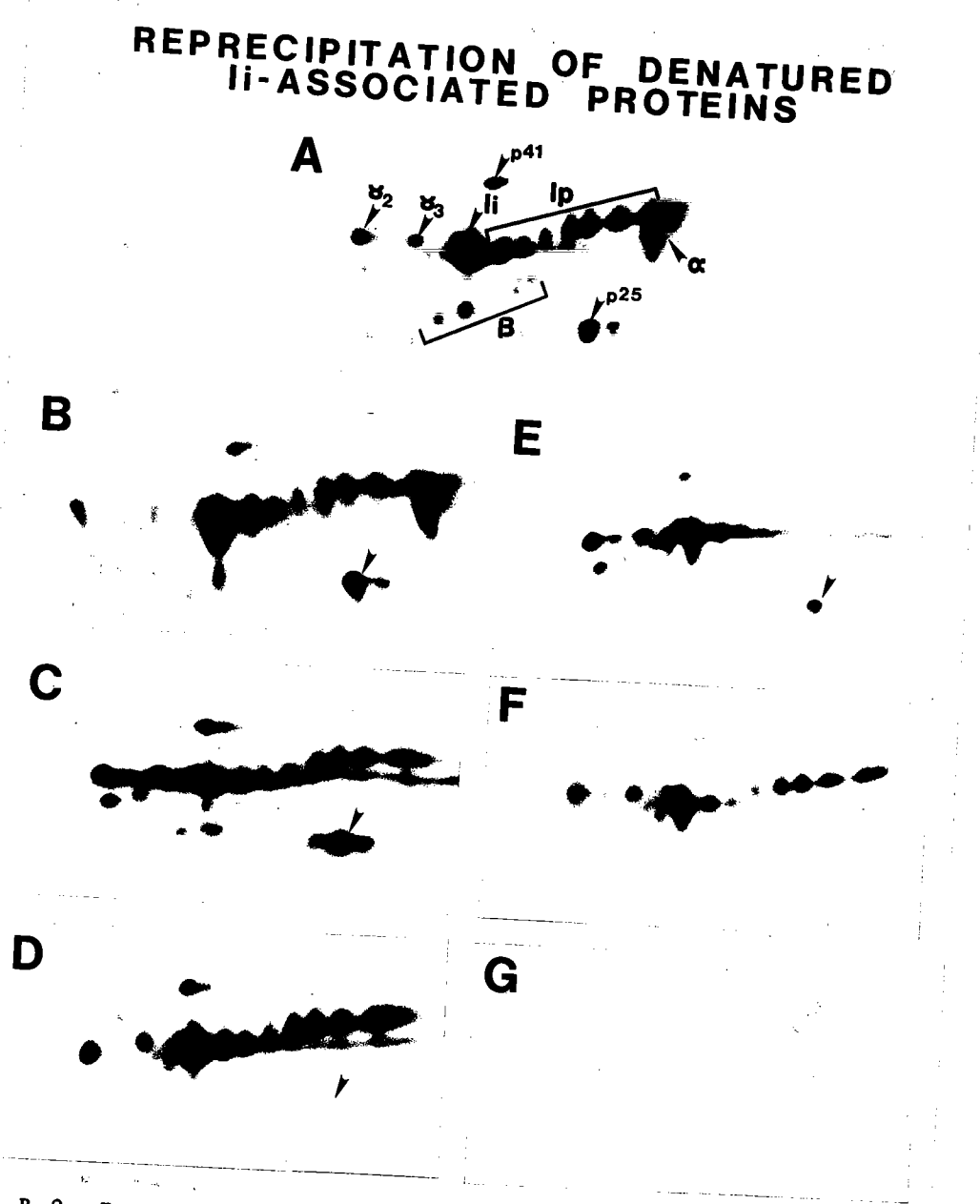


Fig. 3.B.2. Reprecipitation of I_i-associated, denatured proteins. Detergent-solubilized, [^{35}S]methionine-labeled, microsomal membranes of lysed Raji cells were immunoprecipitated with VIC-Y1 mAb (Panel A). VIC-Y1 immunoprecipitated proteins were eluted with 9 M urea and passed over a Sephadex G25 column which was equilibrated in 0.05% Triton, 0.1 M Tris-HCl buffer, pH 8.0 (Panel B). Immunoprecipitates were prepared from the eluted proteins with each of the following antibodies: C351, rabbit heteroantisera to a C-terminal peptide (192-211) of I_i (V. Quaranta, Scripps Clinic and Research Foundation) (Panel C); N350, rabbit heteroantisera to an N-terminal peptide (12-28) of I_i (V. Quaranta) (Panel D); E1, rabbit heteroantisera to a C-terminal peptide (183-193) of I_i (Panel E); VIC-Y1 mAb (Panel F); E4, a rabbit heteroantiserum to p67/69, a molecule not immunoprecipitated by VIC-Y1 (Panel G).

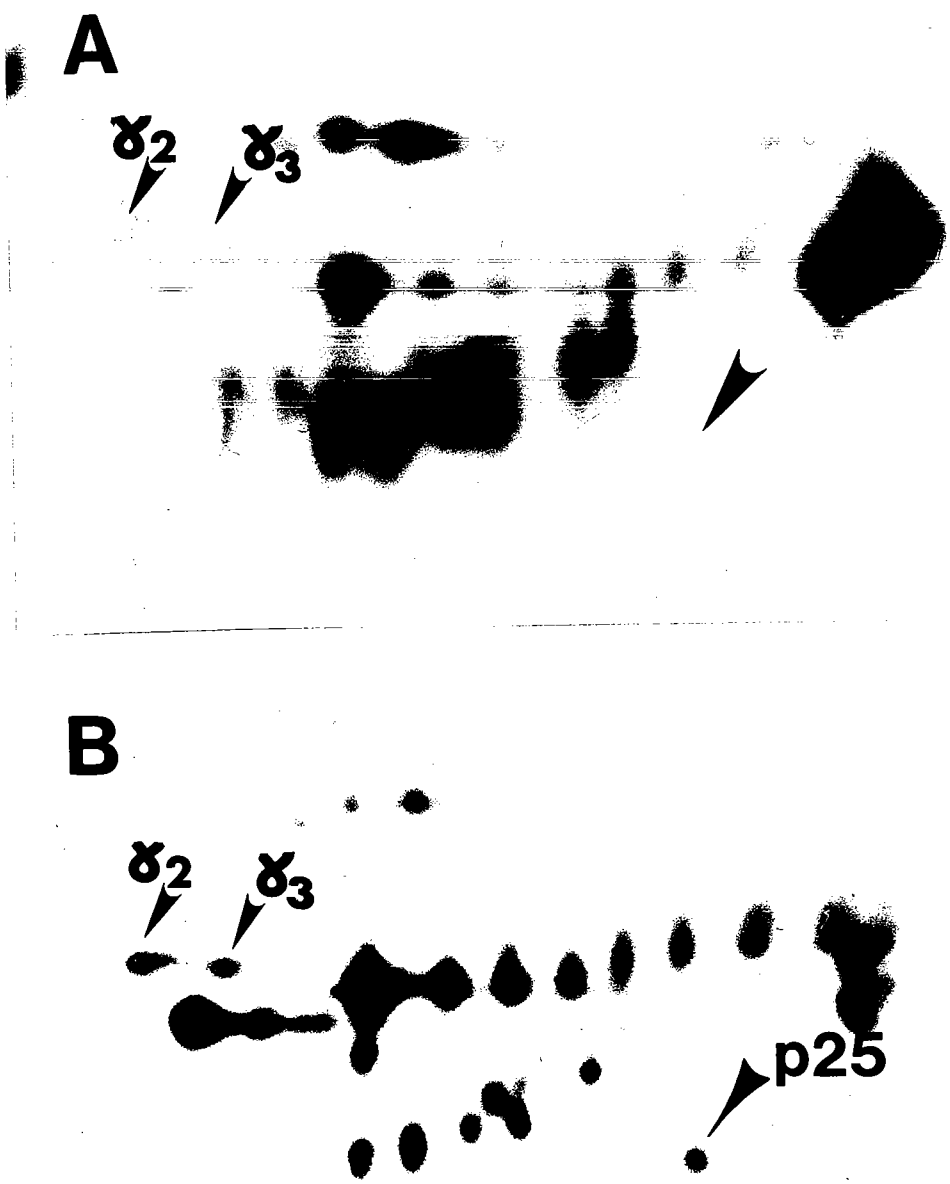


Fig. 3.B.3. Immunoprecipitation of [^{35}S]cysteine-labeled proteins. [^{35}S]Cysteine-labeled (A) or [^{35}S]methionine-labeled (B) proteins were immunoprecipitated with VIC-Y1 anti-I_i mAb from polyclonally *S. aureus*-activated B cells and were subjected to 2-dimensional (nonequilibrium, pH gradient and SDS) electrophoresis. p25 (arrow) was immunoprecipitated from methionine- but not cysteine-labeled cells, while I_i, Ip, p41, γ2, and γ3 were immunoprecipitated from cells with each type of radiolabeling. Illustration courtesy of Dr. Quoc V. Nguyen.

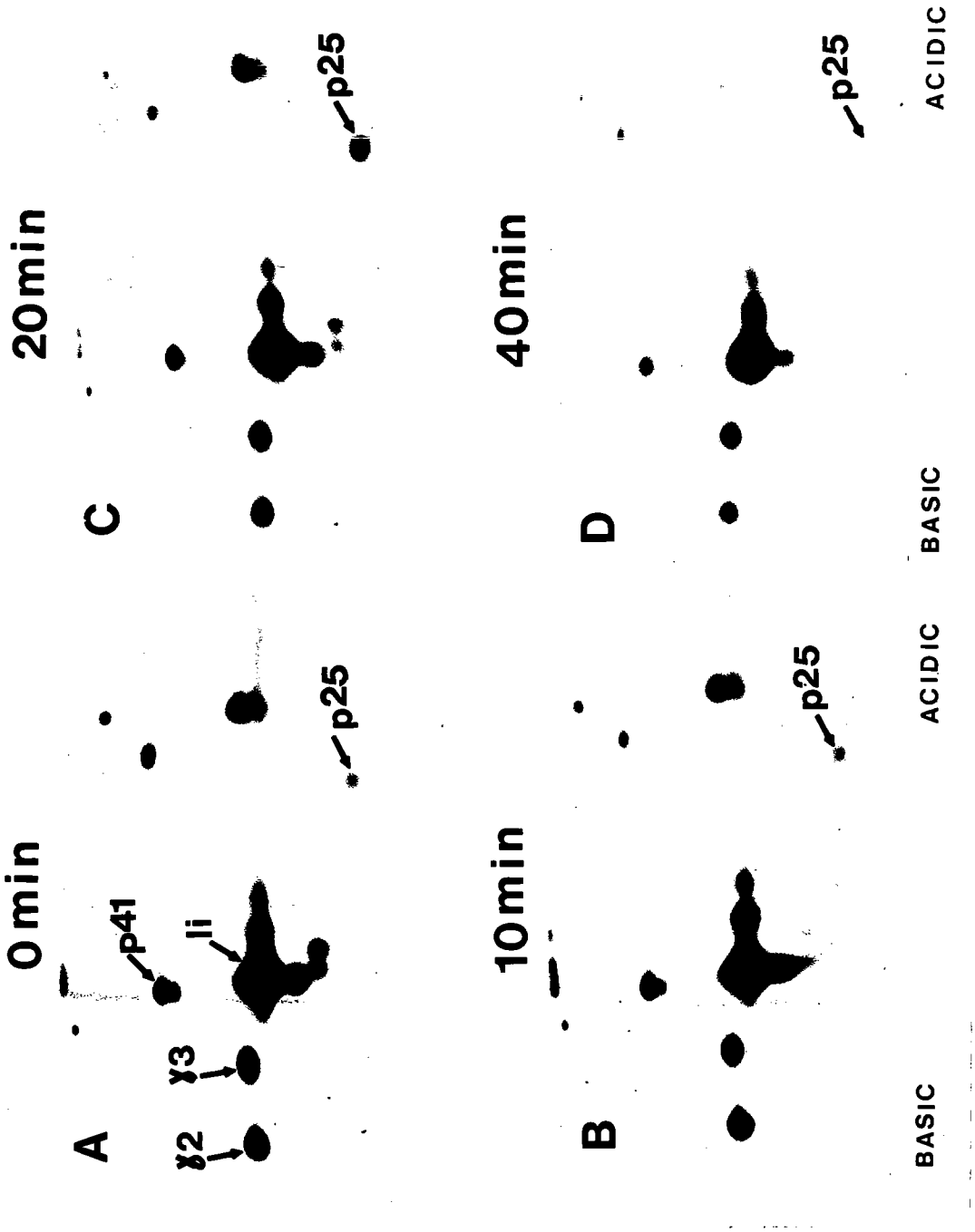


Fig. 3.B.4. Pulse-chase of polyclonally *S. aureus*-activated B cells showing the appearance of p25 maximally 20 min after synthesis. Illustration courtesy of Dr. Quoc V. Nguyen.

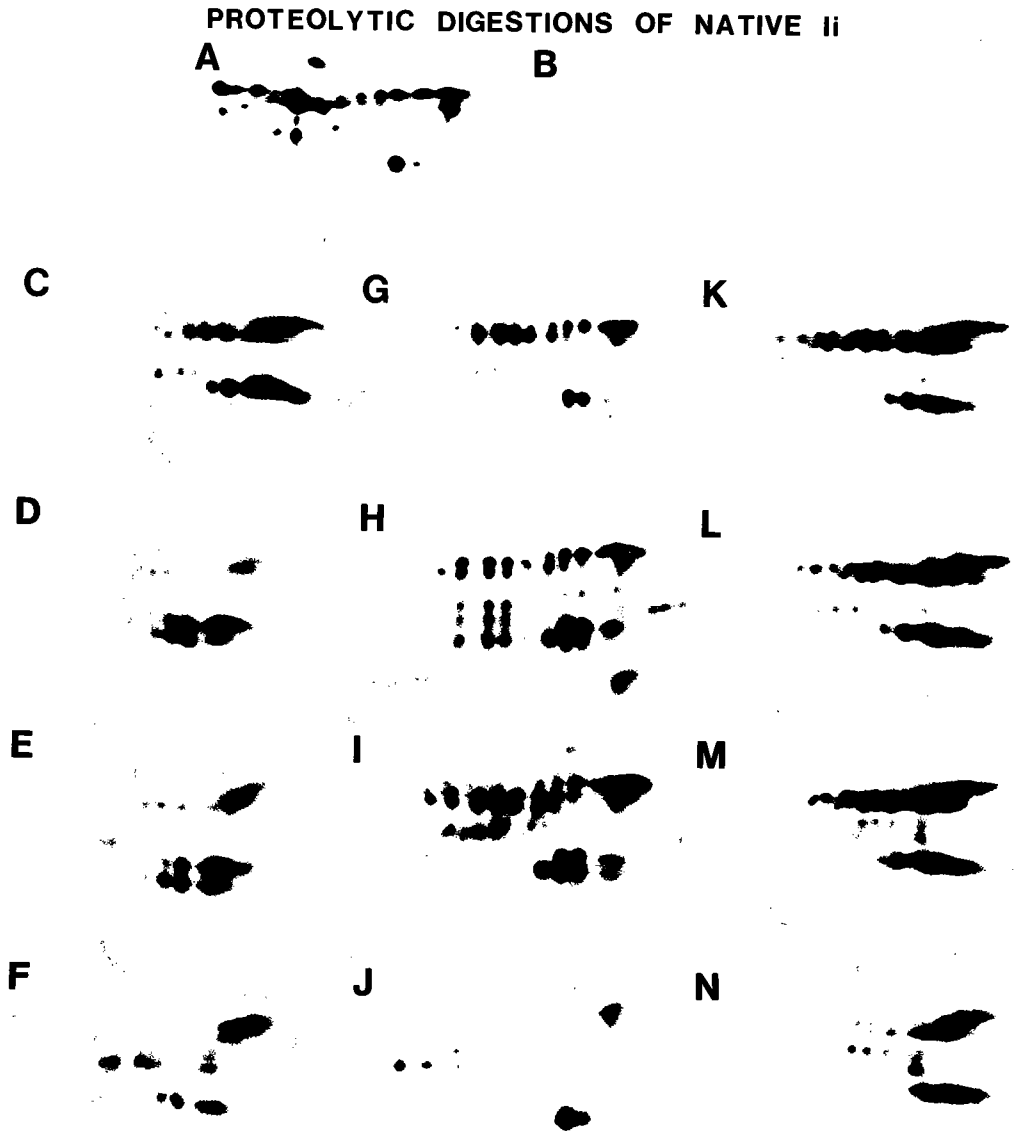


Fig. 3.B.5. Proteolytic digestions of native I_i. Raji cells were metabolically labeled with [^{35}S]methionine, and their membranes were isolated. VIC-Y1, bound to protein A-Sepharose, was used to immunoprecipitate I_i and associated proteins. Eluted antigens were presented in Panel A. A parallel immunoprecipitate with normal mouse ascites was shown in Panel B. Antigens still bound to the antibody-Sepharose matrix were digested by various proteolytic enzymes: Trypsin (C-F), Proteinase K (G-J), and Chymotrypsin (K-N). Enzymes were incubated for various times with antigens: 0 time (C, G, K), 3.5 min (H), 5 min (D, L), 8 min (I), 20 min (E, M), 80 min (F, N), 120 min (J). With each of the enzymes there is a time-dependent build up of products in the area where p25 migrates in a two dimensional gel.

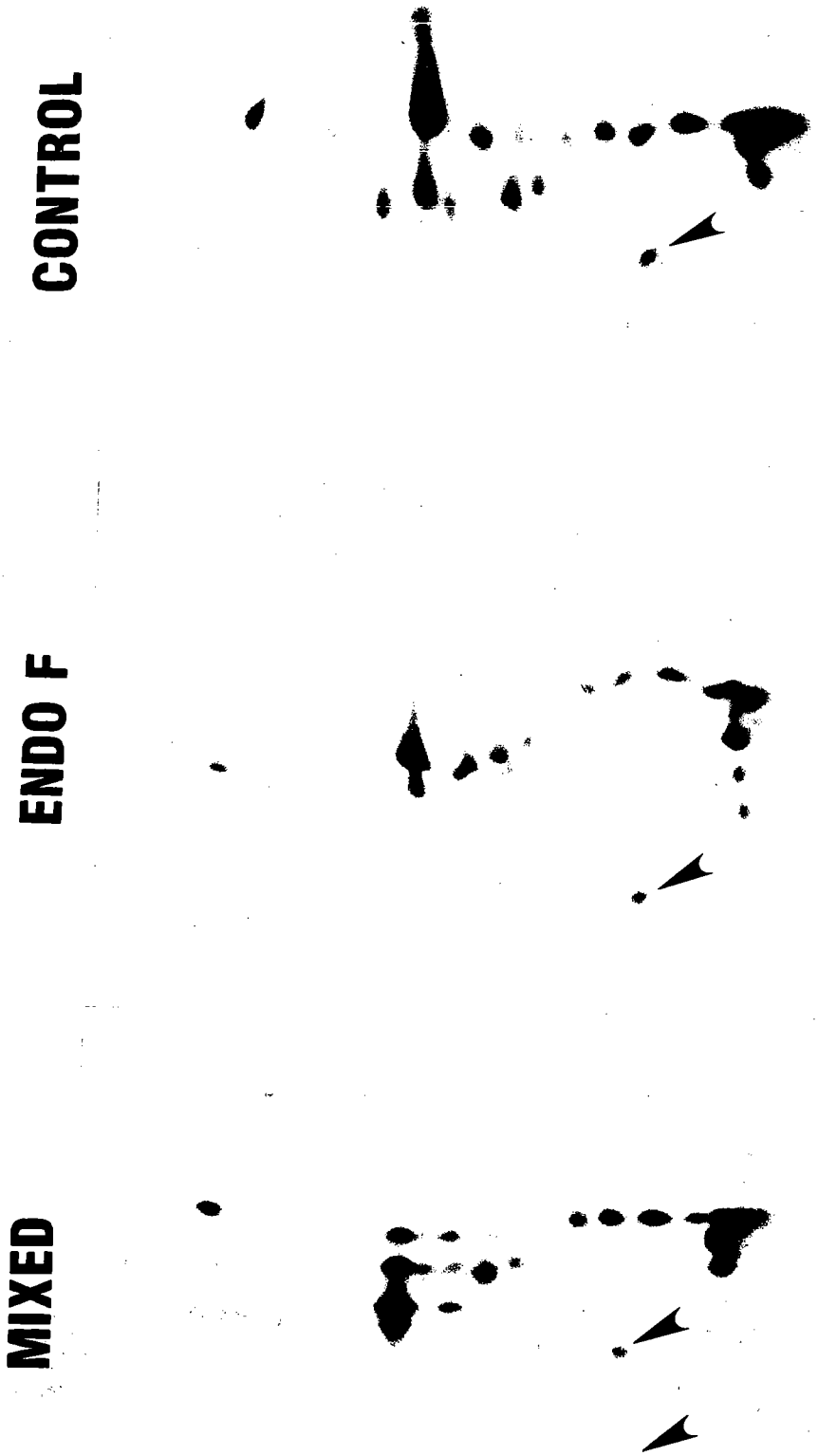


Fig. 3.B.6. Digestion of p25 with endoglycosidase F. "Control" panel shows undigested eluate, "Endo F" panel shows eluate digested with endoglycosidase F, and "mixed" panel shows mixture of undigested and digested proteins.

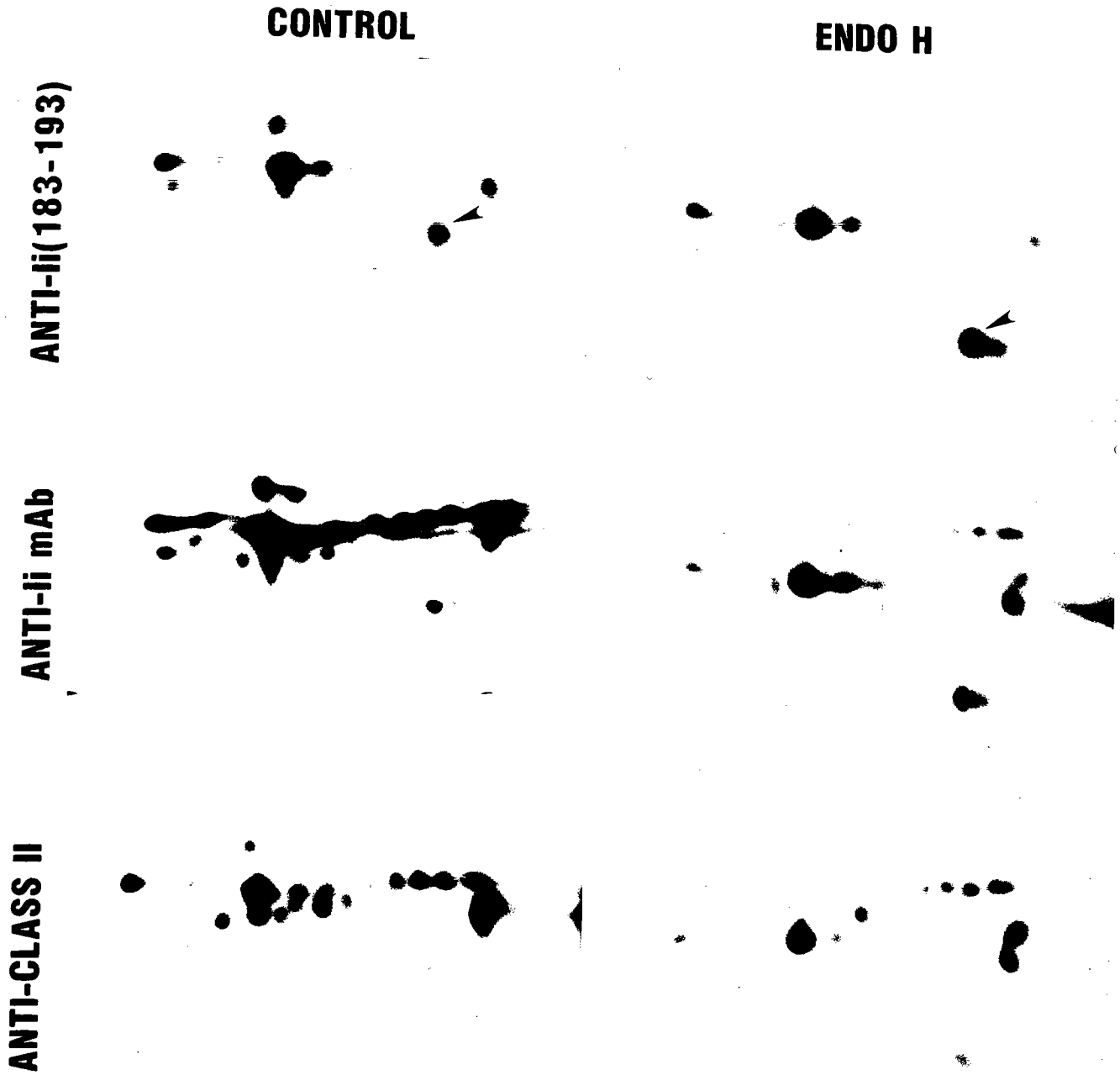


Fig. 3.B.7. Digestion of p25 with endoglycosidase H. "Control" panels show undigested eluate from anti-I$_i$(183-193) serum, anti-I$_i$ monoclonal antibody VIC-Y1, and anti-class II MHC serum. "Endo H" panels show the corresponding eluates digested with endoglycosidase H.

C. Structural Analysis of the Class II MHC Alpha and Beta Chains Leading to a Hypothesis on Their Proteolytic Regulation.

Observation of adjacent of basic amino acids. It was observed that the alpha and beta chains of most HLA-D molecules contained pairs of basic amino acids, lysines and/or arginines, throughout their sequences (Fig. 3.C.1). These pairs of amino acids are well conserved between haplotypes and between species (Figueroa and Klein, 1986).

Arginine and/or lysine pairs have been shown in a variety of systems to be cleaved by a protease. The classic example of such a cleavage being the processing of proinsulin to insulin (Steiner *et al.*, 1969). It can then be hypothesized that proteolytic cleavages of the class II MHC antigens' alpha and beta chains at these sites could be important, in some way, in antigen presentation.

Correlation of cleavage sites to 3 dimensional structure. Brown *et al.* (1988) predicted the structure of the class II desetope by comparison of its sequence to the sequence of the crystallized class I MHC molecule. This predicted structure is shown in Fig. 3.C.2. The walls of the desetope are shown as two extended alpha helices. The placement of these helices is indicated in Fig. 3.C.1. If one looks at the potential cleavage sites, mentioned above, in relation to the structure of the desetope, one sees that these cleavages fall in critical locations. Specifically, if cleavage did occur at these sites

the walls of the desetope could be removed.

Hypothetical cellular functions of proteolytic cleavages of the class II MHC alpha and beta chains. Cleavages at these sites could regulate the creation and/or destruction of the antigen presenting function of the class II MHC proteins. Specifically, proteolytic cleavage of the class II MHC proteins at these sites would be a very efficient way to destroy the antigen presenting capability of the complex. Proteolytic cleavage of the alpha and beta chains might occur while foreign antigen is present, after the complex has reached the cell surface. This would serve to prevent further antigen presentation to T cells after sufficient presentation might have occurred. Alternatively, upon possible release of the foreign peptide, susceptible sites of the class II alpha and beta chains could be cleaved by extracellular proteases, rendering that peptide binding site unable to adsorb, and thus present, ambient peptides. Proteases are known to be released by activated T cells (Pasternack and Eisen, 1985; Pasternack *et al.*, 1986). After internalization, proteolytic cleavage of the alpha and beta chains could occur to inactivate the class II complex. This could serve as a mechanism to destroy complexes that have presented foreign antigen. Furthermore, if I_i is required to catalyze foreign peptide binding to class II MHC molecules, these recycled surface molecules, void of I_i, might bind such peptides inefficiently. Another possibility is that the inactivation of class II MHC molecules could be a mechanism to limit the number of class II

MHC molecules that reach the cell surface.

Alternatively, or in addition, these proteolytic cleavages could facilitate desetope conversion (Guillet *et al.*, 1987). Specifically, excision of the beta chain third hypervariable region between Lys_{71}-Arg_{72} and Arg_{93}-Arg_{94} or the alpha chain hypervariable region between Lys_{48}-Arg_{49} and Lys_{79}-Arg_{80} could permit the binding of structurally similar foreign peptides in the endosome containing foreign antigen, in a process of desetope conversion.

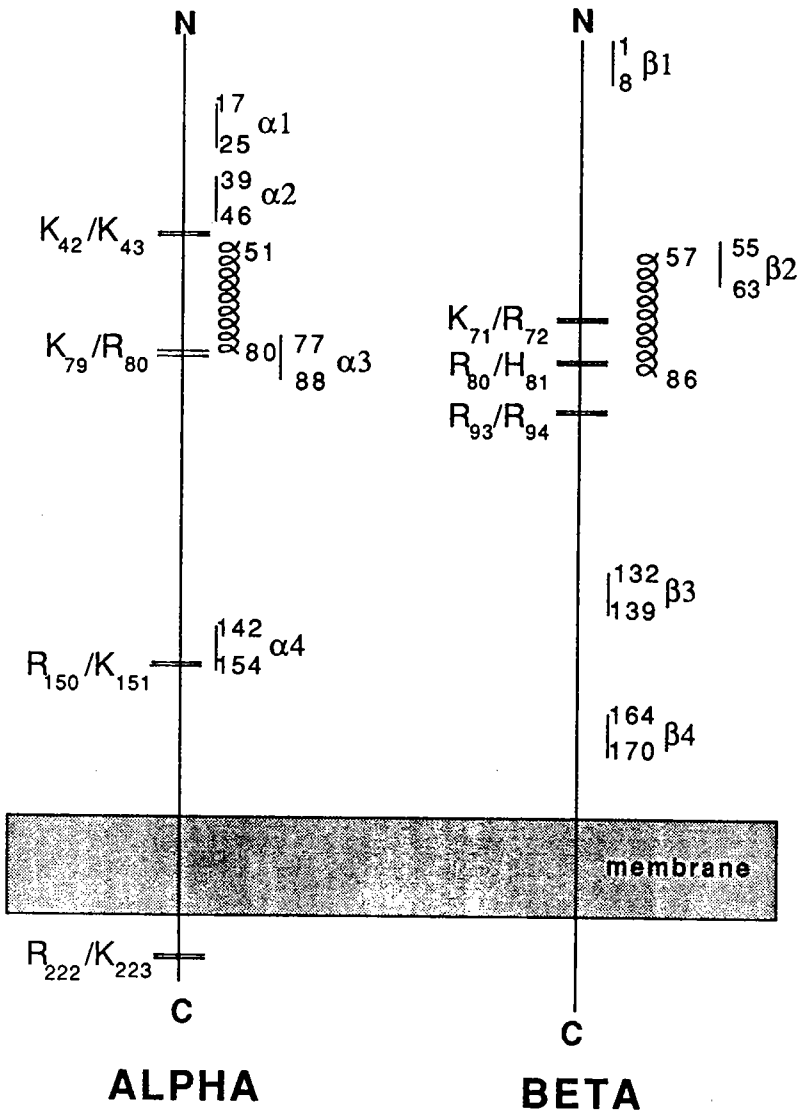


Fig. 3.C.1. Putative cleavage sites on the class II MHC alpha and beta chains. Pairs of basic amino acids about the class II MHC desetope could lead to cleavages which eliminate antigen-presenting function. The putative walls of the class II MHC desetope are shown as helices [α(51-80), β(57-86)]. The peptides synthesized are also indicated.

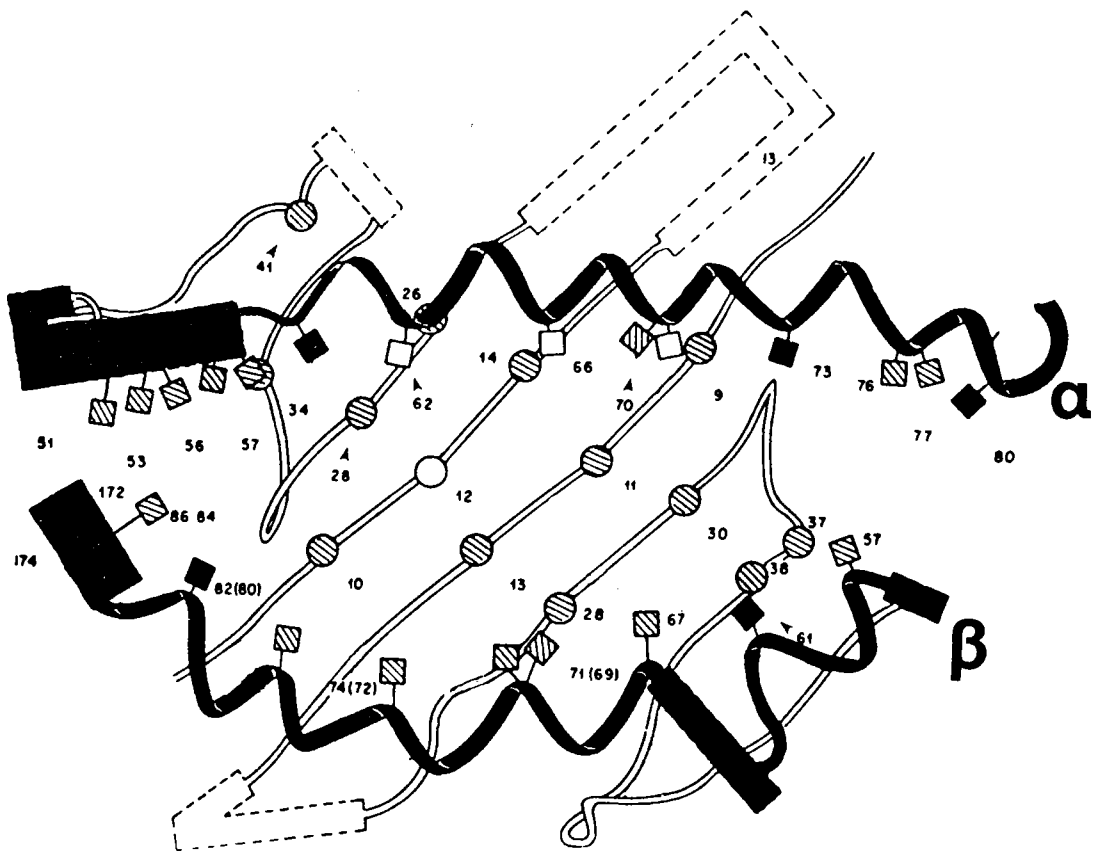


Fig. 3.C.2. Hypothetical structure of the class II MHC desetope (Brown *et al.*, 1988).

D. Proteolysis of the HLA Class II MHC Alpha and Beta Chains

Introduction. Our goal in the following experiments was to demonstrate if the class II MHC alpha and beta chains undergo proteolytic cleavage, and if this cleavage is relevant to the control of antigen processing and presentation. Towards that goal we identified pairs of basic amino acids at structurally prominent sites in the Brown-Wiley model of the class II MHC structure (Brown *et al.*, 1988), demonstrated the existence of class II MHC molecule bound polypeptides in immunoprecipitates by anti-class II sera, and presented strong evidence that these polypeptides were derived from the alpha and beta chains. To achieve this last aim we used western blotting with antisera to synthesized peptides whose sequence was from putatively immunodominant sections of the alpha and beta chains.

Finding endogenous polypeptides bound to class II MHC proteins. In immunoprecipitates, with anti-class II MHC serum, of [^{35}S]methionine-labeled Raji cells, a polypeptide of about 8.4 kD was seen in reduced samples. This band was not seen in the samples that were not reduced or in precipitates with NRS (Fig. 3.D.1). This gel was 10% acrylamide/8 M urea, with an SDS/Tris buffer of pH 6.8. This polypeptide had not been seen previously, however in the 10% gels that are used routinely, polypeptides of about 8.4 kD (about 75 amino acids) are not resolved. This polypeptide is then a portion of some

protein (or a protein) immunoprecipitated with anti-class II MHC serum. It has about 75 amino acids (or less if it has carbohydrate) and is linked to a larger molecule by a disulfide bond. I_i, as a source of this polypeptide, can be ruled out, since it has only one cysteine, and no disulfide linkages. Upon examination of sequences of the alpha and beta chains, this polypeptide could be the N-terminal portion of the beta chain, with a cleavage around Lys_{71}-Arg_{72}. Under non-reducing conditions it might still be connected to the rest of the molecule through a disulfide bond, through Cys_{15}, which is broken under reducing conditions.

Observation of four polypeptides in [^{35}S]cysteine-labeled Vavy cells. The above experiment was with the Raji cell line. Interpretation of results with this cell line could be difficult because Raji cells are heterozygous at the DR locus (DR 3,6) and therefore probably at the DP and DQ loci. Further, the DP and DQ haplotypes of Raji have not been reported. Subsequent experiments have been done with the homozygous cell line, Vavy which is DR 3, DQ 1, DP 2, with each of these molecules being of published sequence (Figueroa and Klein, 1986). Also cells have been labeled with [^{35}S]cysteine rather than [^{35}S]methionine since these residues are more conserved, and upon examination of the sequences of the putative polypeptides (portions of the chains between basic amino acid pairs) cysteines are usually present. Further, since I_i has only one cysteine in its sequence, labeling cells with [^{35}S]cysteine will mask all but

possibly one polypeptide from I_i. Consequently, the polypeptides seen with [^{35}S]cysteine-labeling will be predominantly from the chains of interest, alpha and beta.

Figure 3.D.2 shows an anti-class II MHC immunoprecipitate of [^{35}S]cysteine-labeled Vavy cells run in a 11-23% acrylamide gradient gel. The first lane is the eluted material without digestion. The columns to the right are the same material digested with trypsin of constant concentration, but with increasing time. Four polypeptides are resolved, at about 14.4, 11.4, 8.4 and 4.0 kD. Since these cleavages are not enhanced with incubation with trypsin, we conclude that a more specific enzyme is used *in vivo*, or that addition controlling factors (*i.e.* desetope full or empty) play a role. The next experimental goal was to determine if these polypeptides were derived from the alpha or beta chains. To do this we attempted to make anti-peptide antibodies to the alpha and beta chains and western blot them to these polypeptides.

Production of anti-beta chain and anti-alpha chain antibodies. To produce antibodies made to specific portions of the alpha and beta chains, we selected four peptides from the DR 3 beta chain and four peptides from the DR 3 alpha chain sequences using a Hopp-Woods type of 9 amino acid window hydrophobicity program generated in the lab. The peptides were called β1, β2, β3, β4, and α1, α2, α3, and α4. Their sequences are shown in Fig. 3.D.3 and 3.D.4, respectively. The peptides were also selected such that (1) the peptides were from

different regions of the molecules so that they might recognize different putatively cleaved polypeptides and (2) antibodies to the different peptides might selectively recognize different isotypes of the HLA-D complexes present in Vavy cells. For example, the peptides to the beta chain consisted of: (1) one to a C-terminal sequence conserved in all HLA-D isotypes, (2) one to a C-terminal sequence common to only HLA-DR molecules, (3) one to a more central sequence that is common to all HLA-D isotypes and (4) one to a central portion of HLA-DR antigens (Fig. 3.D.3). Similarly, a comparison of the alpha chain peptides to the HLA-D isotypes present in Vavy cells is shown in Fig. 3.D.4. The eight peptides were synthesized, coupled to KLH, and injected into rabbits (Fig. 3.D.5). Sera were taken from the animals.

Characterization of the anti-peptide sera. To determine if the rabbits injected with the various peptides were making antibodies to the peptide, which reacted with native or denatured proteins, we formulated a multistep testing protocol, detailed below. The results of this regime are summarized in Fig. 3.D.5.

Dot blots to characterize anti-beta chain sera. To assay the sera for antibodies to the injected peptides, a dot blot assay was initially used, as shown in Figures 3.D.6 to 3.D.9. Peptide was coupled to BSA and blotted on a sheet of nitrocellulose. Pieces were incubated with various dilutions of the sera, washed, and further

incubated with [^{125}I]protein A. The assay worked well to screen out non-productive animals (those injected with peptides β2 and β4), but was relatively peptide non-specific for the animals that gave a positive result. That is, sera from the animals injected with the peptides β1 and β3 bound to the peptide-BSA conjugate in an antibody dose dependent fashion. However, this titration also occurred to an irrelevant peptide-BSA conjugate. To identify if the animals were making specific antibody we decided to use an ELISA, which (1) being more quantitative, could discriminate positive responses above nonspecific background reactions and, (2) could use uncoupled peptide, to eliminate nonspecific binding.

ELISA to characterize anti-alpha and beta chain sera. Sera from the rabbits injected with the peptides β1 and β3, as well as those rabbits injected with the four alpha chain peptides, were tested by ELISA. In these assays unconjugated peptide was deposited on the wells of a microtiter plate and exposed to the sera from the respective rabbits. A second antibody, a horseradish peroxidase coupled goat anti-rabbit antiserum, was used and specific binding was detected with enzyme-produced colored product [the substrate was ABTS, 2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonate)]. The results were read with a microtiter plate reader. The results of these experiments are shown in Fig. 3.D.10 to 3.D.13. The animals injected with the peptide α3 ($\alpha 3^a$ and $\alpha 3^b$) both showed specific reactivity to the peptide (Fig. 3.D.10 and 3.D.11, respectively). One animal injected

with the peptide α4 ($\alpha 4^a$) also showed antibody production specific to the injected peptide (Fig. 3.D.12). All of the other animals tested did not show reactivity to the specific peptide greater than to an irrelevant peptide or the preimmunization sera, including the animals injected with β2 and β4. An example of these negative results is shown in Fig. 3.D.13.

Immunoprecipitates to characterize anti-alpha and beta chain sera. To address the question if the antibodies produced could recognize alpha chains isolated from cells, immunoprecipitations of [^{35}S]methionine-labeled Vavy cell microsomal membranes in the native and denatured state were attempted. Known positive controls were also used. The results of these experiments are shown in Fig. 3.D.14. The migration of immunoprecipitated material was identified by molecular weight and by comparison to known alpha and beta chains. Rabbit $\alpha 3^b$ (to peptide α3) precipitated two bands, at about 35 and 32 kD. The 35 kD band is the expected weight of the alpha chain. The 32 kD band could be the unprocessed form of the alpha chain. Alternatively this 32 kD species could be I_i that was in an I_i-alpha chain complex that was resistant to the denaturation procedure.

Western blots to characterize anti-alpha and beta chain sera. To address whether these antisera could western blot to electrophoresed, electrotransferred alpha chains, as would be necessary for them to be

used in the following experiments, this ability was assayed. Microsomal membranes from both Raji and Vavy cells were produced without radioactive label and electrophoresed in a 10% acrylamide SDS gel. The proteins were transferred to nitrocellulose, and strips were incubated with dilutions of the antisera. The adherent antibodies were detected with [^{125}I]protein A (Fig. 3.D.15). The blot of the anti-23,30 (anti-class II) serum indicated the position of the beta chain, about 27 kD, as marked by the arrowhead. The $\alpha 3^a$ antiserum blotted to a protein that was about the molecular weight of alpha chain, while the $\alpha 4^a$ did not. In other experiments specific binding was detected colorimetrically, using an HRP-goat-anti-rabbit conjugate. Those experiments, also using preimmunization sera, confirmed $\alpha 3^a$ and $\alpha 3^b$ both blot to a band that is probably the class II MHC alpha chain. That data are not shown, however Fig. 3.D.16, Panel D, shows similar results.

Immunoblots of [^{35}S]cysteine labeled polypeptides. To show definitively that the polypeptides were derived from the alpha chains, we used the anti-alpha chain sera to blot to these polypeptides. Specifically, Vavy cells were labeled with [^{35}S]cysteine and immunoprecipitated with anti-class II MHC serum. The immunoprecipitated proteins were electrophoresed in a 17-27% gradient gel system (Fig. 3.D.16). One portion of the gel was saturated with fluor and autoradiographed to determine if the polypeptides in question were present. Panel B shows two small polypeptides at about

15.5 and 13.5 kD and probably a third polypeptide at about 12 kD (indicated by arrowheads). Panel A is a lighter exposure of this piece of the gel, to demonstrate the major species and the molecular weight standards. The rest of the gel, containing [^{35}S]cysteine-labeled anti-class II serum immunoprecipitated proteins, was electroblotted to nitrocellulose. After blotting, the gel was autoradiographed (Panel C). The nitrocellulose was probed with antisera and the respective preimmunization sera (Panel D), and specific binding was detected colorimetrically, using an HRP-goat-anti-rabbit conjugate. $\alpha 3^a$ and $\alpha 3^b$ sera recognize the class II alpha chain (immunoprecipitated previously by an anti-class II MHC serum), as indicated by arrowheads. $\alpha 3^b$ antiserum blotted to a band in the range of the observed polypeptides (about 12-16 kD). The problem here is that there was an inadequate separation of the polypeptides in this gel to determine if: (1) there were 2 or 3 species and (2) to which specific band the antiserum blotted. Also since this polypeptide is a minor species, and HRP was used to detect antibody binding, there was a problem with adequate colorimetric intensity of the blotted band.

To better visualize the blotted bands, better separate the polypeptides, and to try to resolve the question of comigration of bands, the above experiment was repeated on a large 11-23% gradient gel system, and adherent antibodies were detected with [^{125}I]protein A (Fig. 3.D.17). Also in this experiment [^{35}S]methionine-labeled Vavy cell membranes, immunoprecipitated with anti-class II MHC antibodies, were run. In Panel A, both the [^{35}S]methionine and [^{35}S]cysteine-labeled immunoprecipitates include the alpha and beta

chains. This piece of the gel was exposed to film longer (Panel B) to demonstrate the [^{35}S]methionine-labeled material included p25 and a polypeptide of about 12.5 kD. In comparison, the [^{35}S]cysteine-labeled immunoprecipitate did not show p25, but did show three polypeptides, of about 15.5, 12.5, and 10 kD. The other side of this gel, which had a continuous trough of [^{35}S]cysteine-labeled immunoprecipitate run into the gel, was electroblotted to nitrocellulose. Strips of this nitrocellulose was probed with various antibodies, and preimmunization sera. The bound antibody was detected with [^{125}I]protein A and the nitrocellulose strips were exposed to film (Panel C). As a control, a piece of nitrocellulose with only the blotted proteins was exposed with the immunoblots to film for the same length of time, to determine the extent of exposure from the ^{35}S. The comparison of the autoradiograph of this strip (labeled "No Ab"), to the blotted strips indicated the amount of film exposure from ^{35}S, and also the positions of the proteins transferred to the nitrocellulose. In the case of the western blots with $\alpha 3^{a}$ and $\alpha 3^{b}$ sera, there is an enhancement of the alpha chain band indicating exact comigration of the blotted band with the [^{35}S]methionine-labeled alpha chain band. This is very good evidence of recognition of the alpha chain with this antisera. The antiserum $\alpha 3^{b}$ blotted to a band in the range of the polypeptides. In Fig. 3.D.18 the $\alpha 3^{b}$ blot is shown again with a longer exposure to the film, along with a longer exposure of a strip of unprobed nitrocellulose, containing the [^{35}S]cysteine-labeled, blotted proteins. The band recognized by $\alpha 3^{b}$ lines up with

[^{35}S]cysteine-labeled, anti-class II MHC immunoprecipitated polypeptide. In this figure the unprobed strip was exposed to film about three times longer than the $\alpha 3^{b}$ blotted strip. This indicates that the intensity of the [^{35}S]cysteine-labeled, unprobed band is greatly enhanced by the $\alpha 3^{b}$ antibody probing and detection with [^{125}I]protein A. Consequently, there is a specific recognition of the $\alpha 3^{b}$ serum for this polypeptide. This band is probably the most intense of the three polypeptides, the 12.5 kD polypeptide. It can be concluded that the $\alpha 3^{b}$ antiserum recognizes one of these polypeptides, and presumably this polypeptide contains at least some portion the alpha chain sequence 77-88.

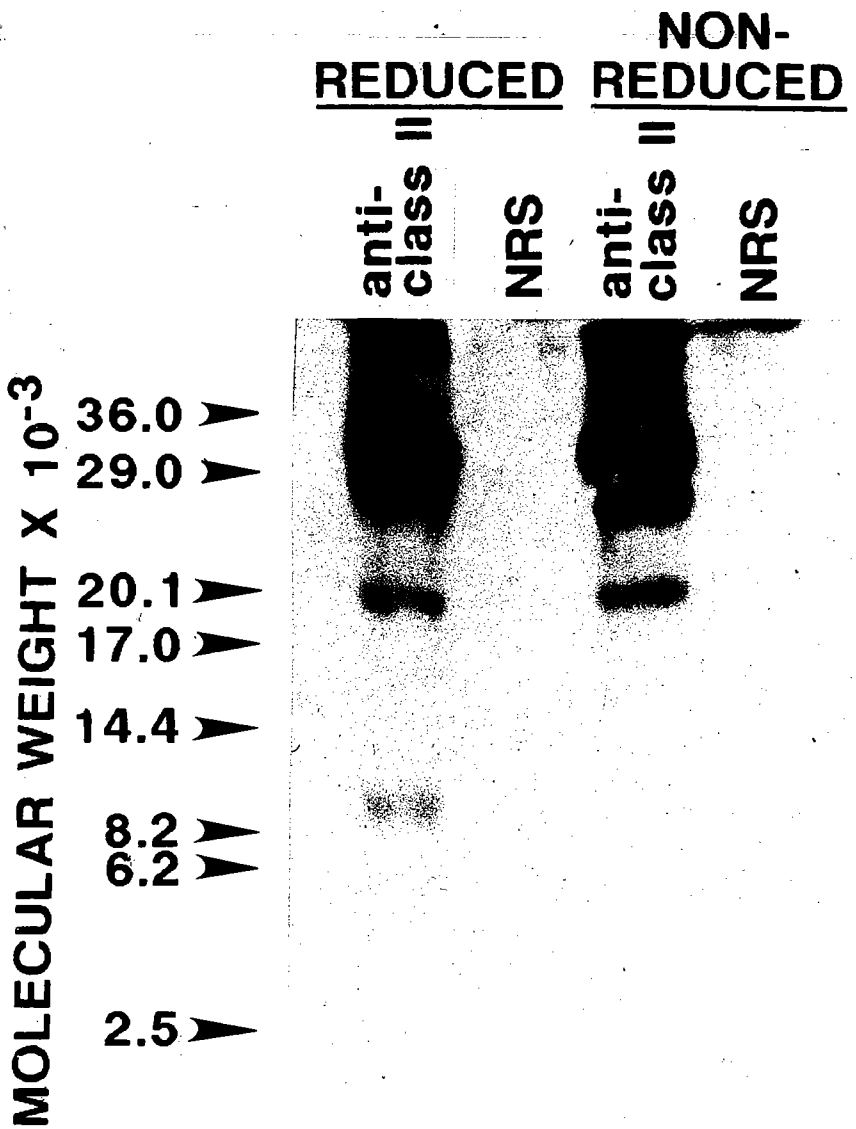


Fig. 3.D.1. Anti-class II MHC serum immunoprecipitates of Raji cell membranes, showing a 8.4 kD band upon reduction.

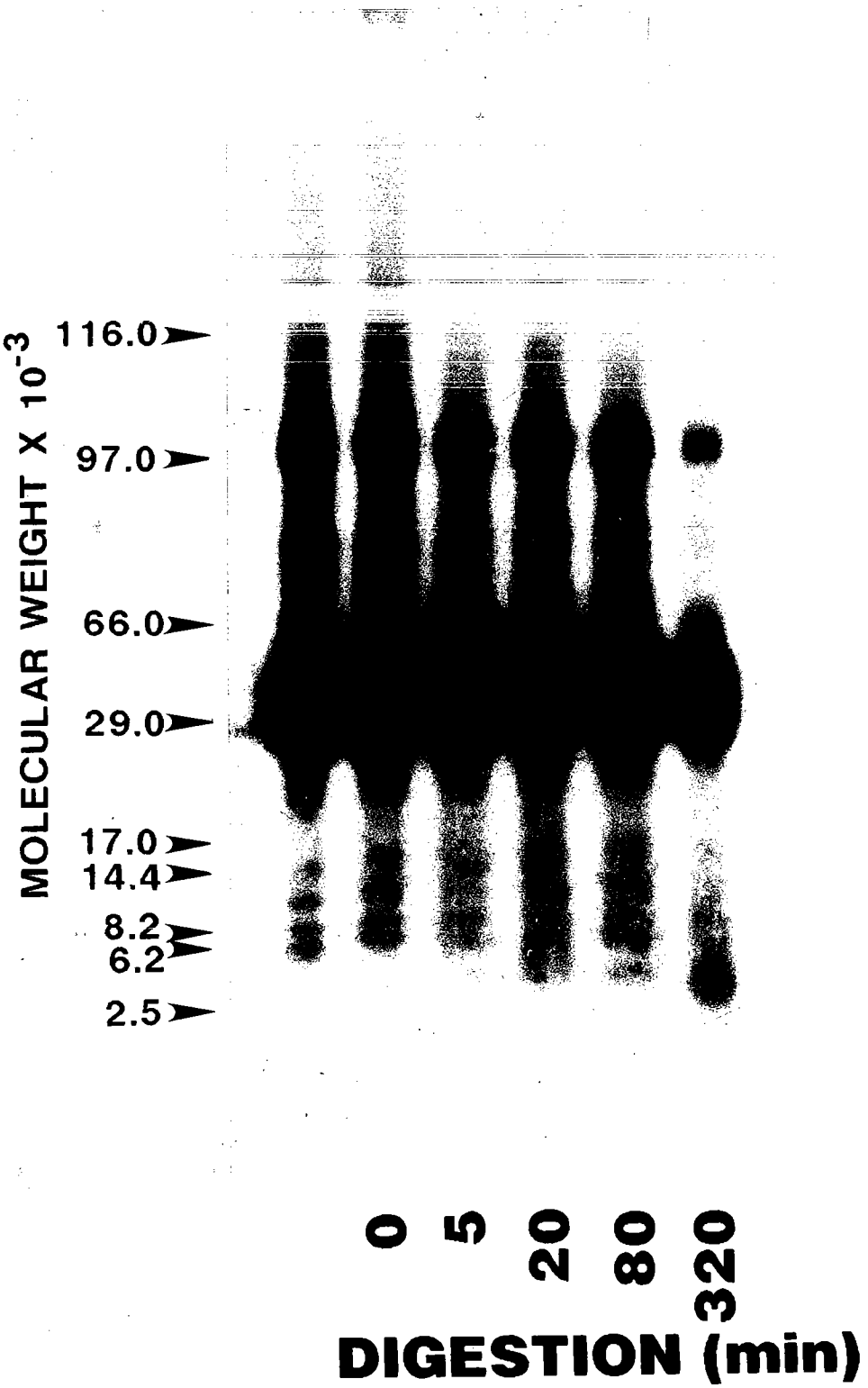


Fig. 3.D.2. Polypeptides associated with class II MHC molecules. Anti-class II MHC serum immunoprecipitates of [^{35}S]cysteine-labeled Vavy cell membranes, electrophoresed on a gradient gel, indicating four polypeptides, which were calibrated at 14.4, 11.4, 8.4 and 4.0 kD.

BETA CHAIN PEPTIDES

	β1	β2	β3	β4
DR BETA 3	GDTRPRFL	RDPAEYWNS	WFRNGQEE	TVPQSGE
DQ BETA 2	RDSPEDFV 25%	RPVAEYWNS 78%	WFRNDQEE 88%	MTPQRGD 43%
DP BETA 1	RATPENTL 25%	RPAAEYWNS 78%	WFLNGQEE 88%	MTPQQGD 43%
	1-8	55-63	132-139	164-170
	DR	DR, DP, DQ	DR, DP, DQ	DR

Fig. 3.D.3. Sequence of peptides made to mimic portions of the DR 3 beta chain, with a comparison to the respective sequence in the DP and DQ molecules present in Vavy cells.

ALPHA CHAIN PEPTIDES

	α1	α2	α3	α4
DR ALPHA 3	YLNPDQSGE	DMAKKETVy	MTKRSNYTPITN	LPREDHLFRKFHY
DQ ALPHA 2	YQSVGPSSQ 22%	DLERKETV 63%	MTKRSNYTPITN 100%	LIKRSNSTAATNY 15%
DP ALPHA 1	VQTHRPTGE 22%	DLDKKETV 75%	LIQRSNHTQATN 50%	LPRTDYSFHKFHY 69%
	17-25	39-46	77-88	142-154
	DR	DR, DP, DQ	DR, DQ	DR, DP

Fig. 3.D.4. Sequence of peptides made to mimic portions of the DR 3 alpha chain, with a comparison to the respective sequence in the DP and DQ molecules present in Vavy cells.

PEPTIDE NAME	SEQUENCE	TYROSINE ADDITION	POSSIBLE ISOTYPE SPECIFICITY	REGION	COUPLER	RABBIT NAME	DOT BLOT	ELISA	WESTERN BLOT		IMMUNPRECIPITATION			
											RAJI		VAVY	
									RAJI	VAVY	NATIVE	DENAT.	NATIVE	DENAT.
β1	1-8		DR	N TERM	EDAC	$\beta1^a$	+	-						
						$\beta1^b$	+	-						
β2	55-63		DR DP DQ	N TERM	TOLIDINE	$\beta2^a$	-							
						$\beta2^b$	-							
β3	131-138		DR DP DQ	MIDDLE	EDAC	$\beta3^a$	+	-						
						$\beta3^b$	+	-						
β4	163-169		DR	MIDDLE	TOLIDINE	$\beta4^a$	-							
						$\beta4^b$	-							
α1	17-25		DR	N TERM	EDAC	$\alpha1^a$		-						
						$\alpha1^b$		-						
α2	39-46	Tyr added C term.	DR DP DQ	N TERM	EDAC	$\alpha2^a$		-						
						$\alpha2^b$		-						
α3	77-88		DR DQ	MIDDLE	EDAC	$\alpha3^a$		+	+	+	-		-	-
						$\alpha3^b$		+	+	+	-		-	+
α4	142-154		DR DP	MIDDLE	EDAC	$\alpha4^a$		+	-	-	-		-	-
						$\alpha4^{b\ DIED}$								
Ii	146-169	Tyr sub. for 146		MIDDLE	TOLIDINE	E3					-			
						E4			-		-			
						E5					-			
						E6					-			
Ii	183-193	Tyr added C term.		C TERM	TOLIDINE	E1	+	+	+/-	+/-	+	+	+	+
						E2					-			
Ii	194-202			C TERM	TOLIDINE	C10	-							
						C11	-							
					EDAC	L6	-							
Ii	203-211			C TERM	TOLIDINE	C12	-							
						C13	-							
					EDAC	L7	-							

Fig. 3.D.5. Chart of anti-alpha and anti-beta chain peptide antisera with the results of various characterization methods.

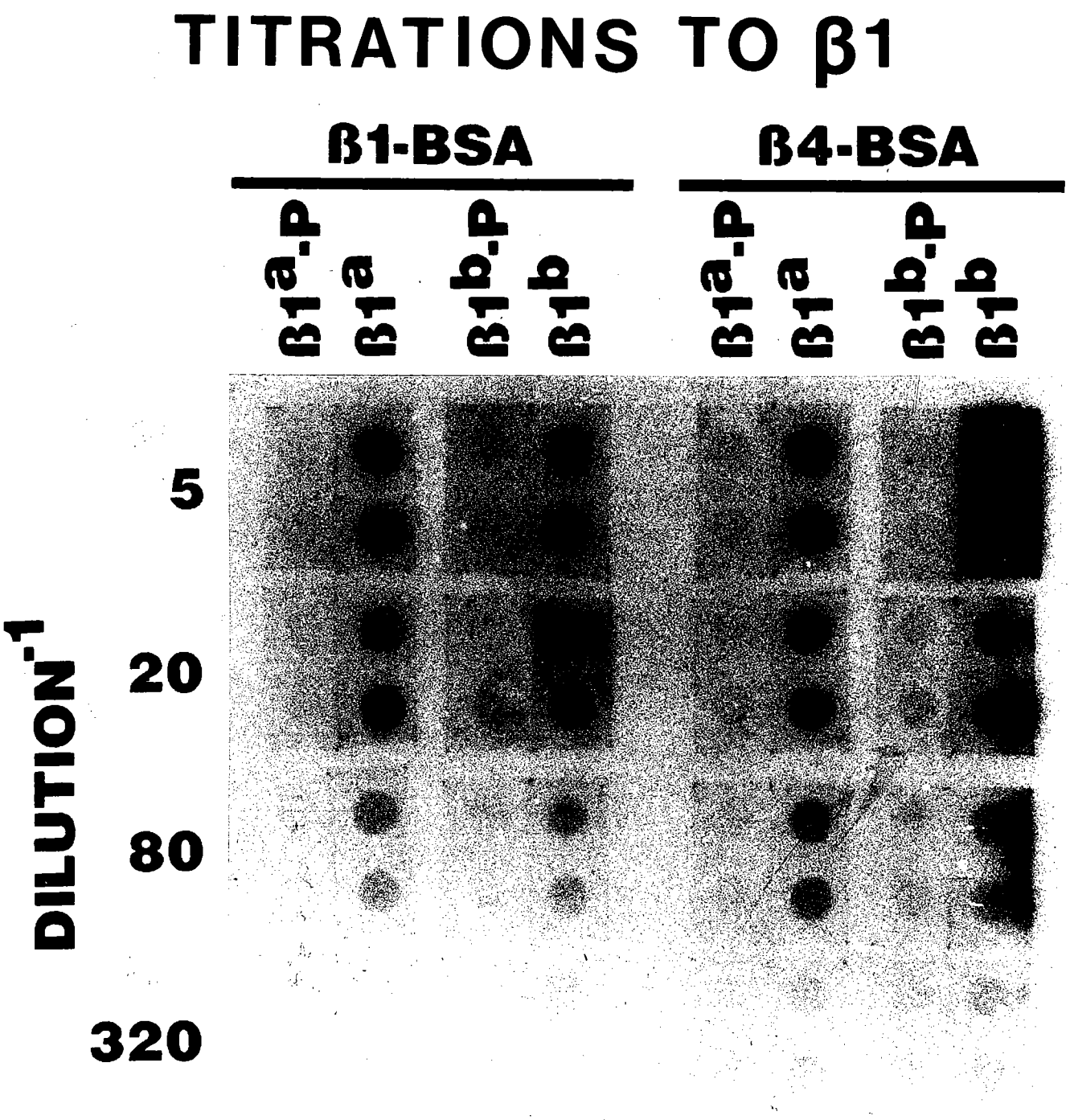


Fig. 3.D.6. Dot blot titers of $\beta 1^a$ and $\beta 1^b$, with preimmunization sera (-P), to the respective and an irrelevant peptide-BSA conjugate.

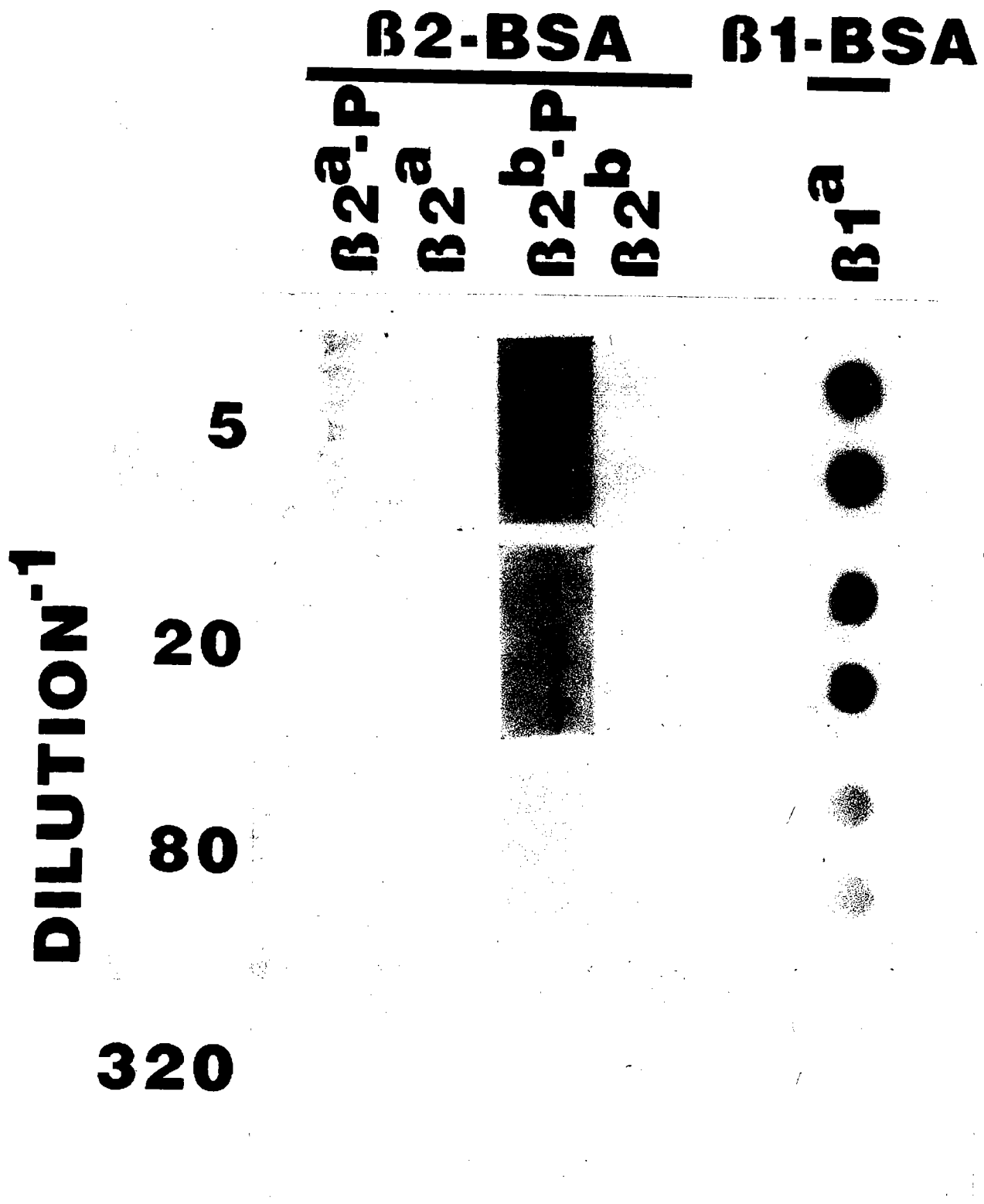


Fig. 3.D.7. Dot blot titers of $\beta 2^a$ and $\beta 2^b$, with preimmunization sera (-P), to the respective and an irrelevant peptide-BSA conjugate.

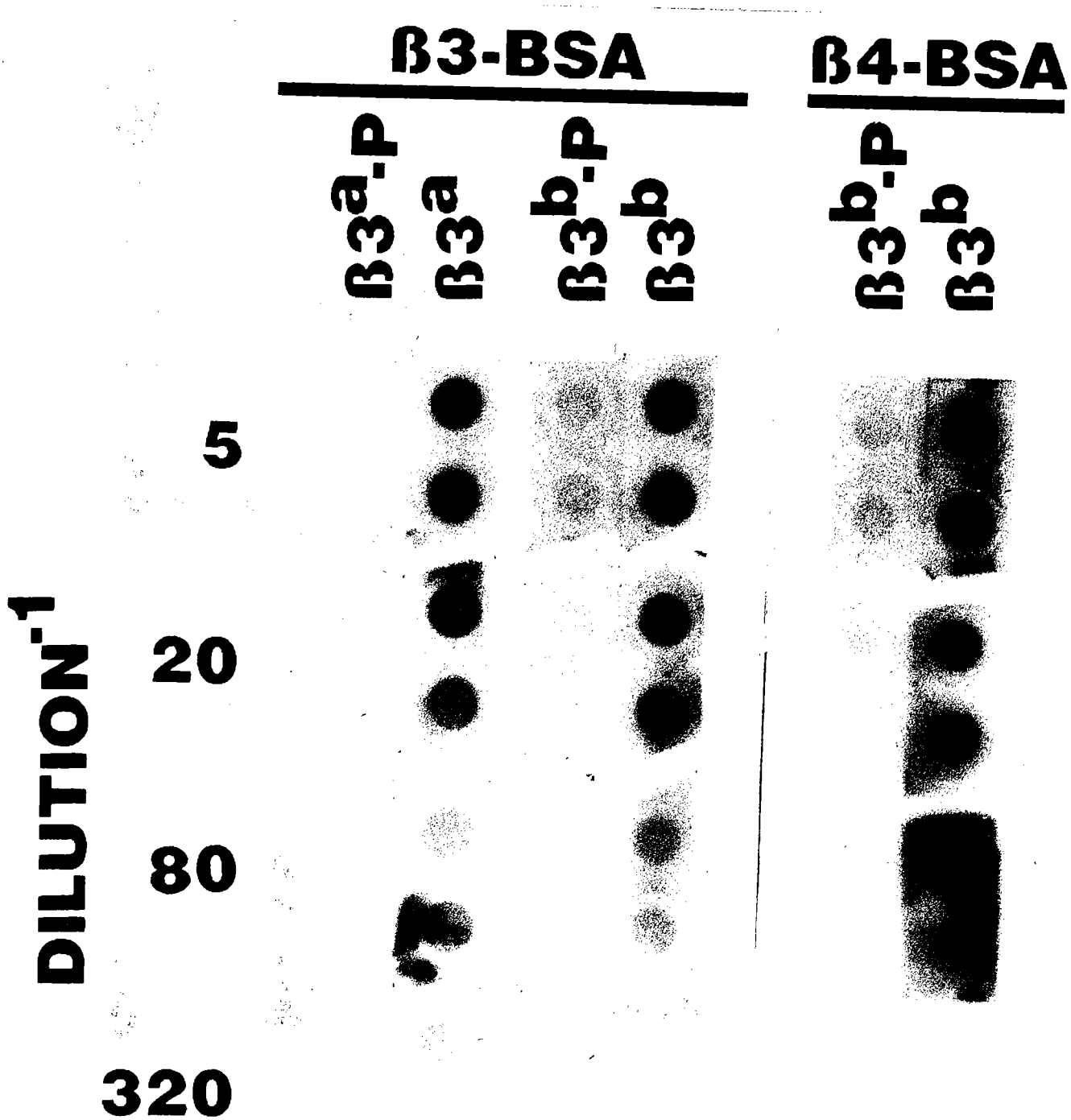


Fig. 3.D.8. Dot blot titers of $\beta 3^a$ and $\beta 3^b$, with preimmunization sera (-P), to the respective and an irrelevant peptide-BSA conjugate.

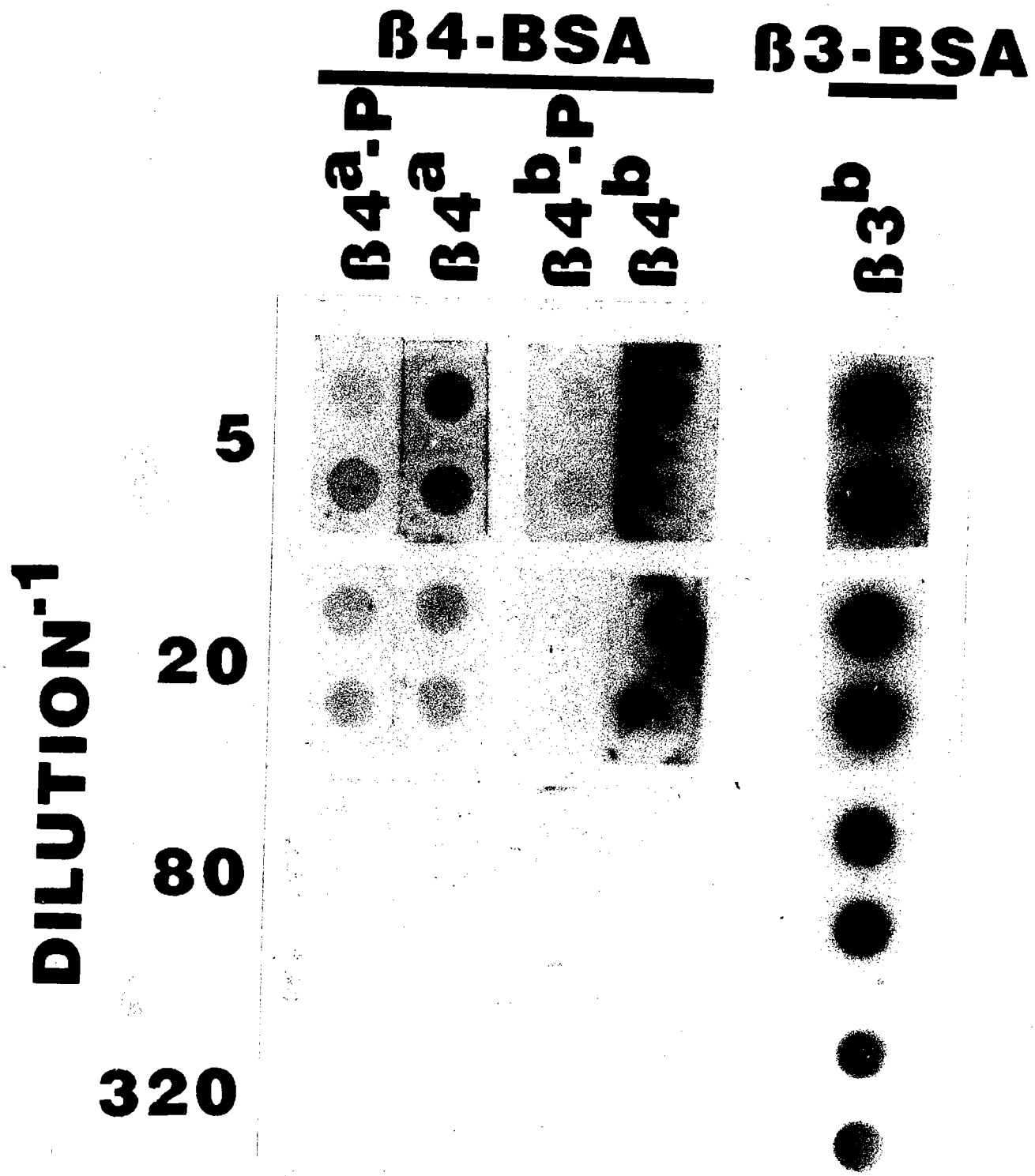


Fig. 3.D.9. Dot blot titers of $\beta 4^a$ and $\beta 4^b$, with preimmunization sera (-P), to the respective and an irrelevant peptide-BSA conjugate.

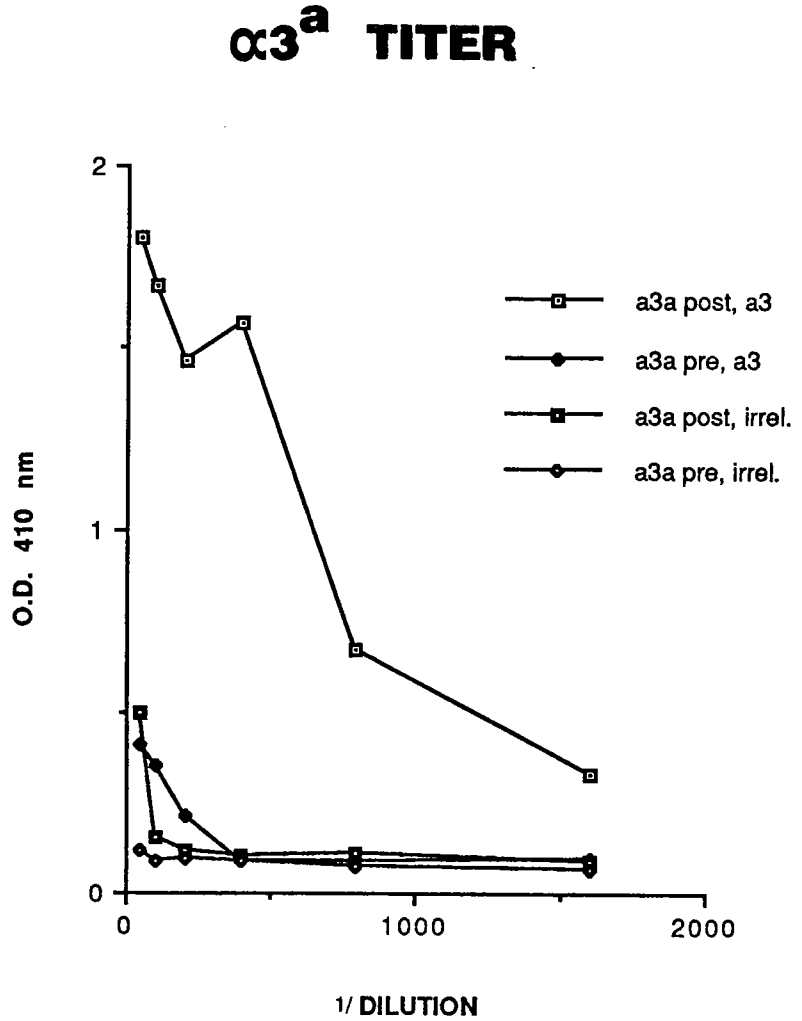


Fig. 3.D.10. ELISA titer of antiserum $\alpha 3^a$, with preimmunization and postimmunization sera, against the respective and an irrelevant peptide.

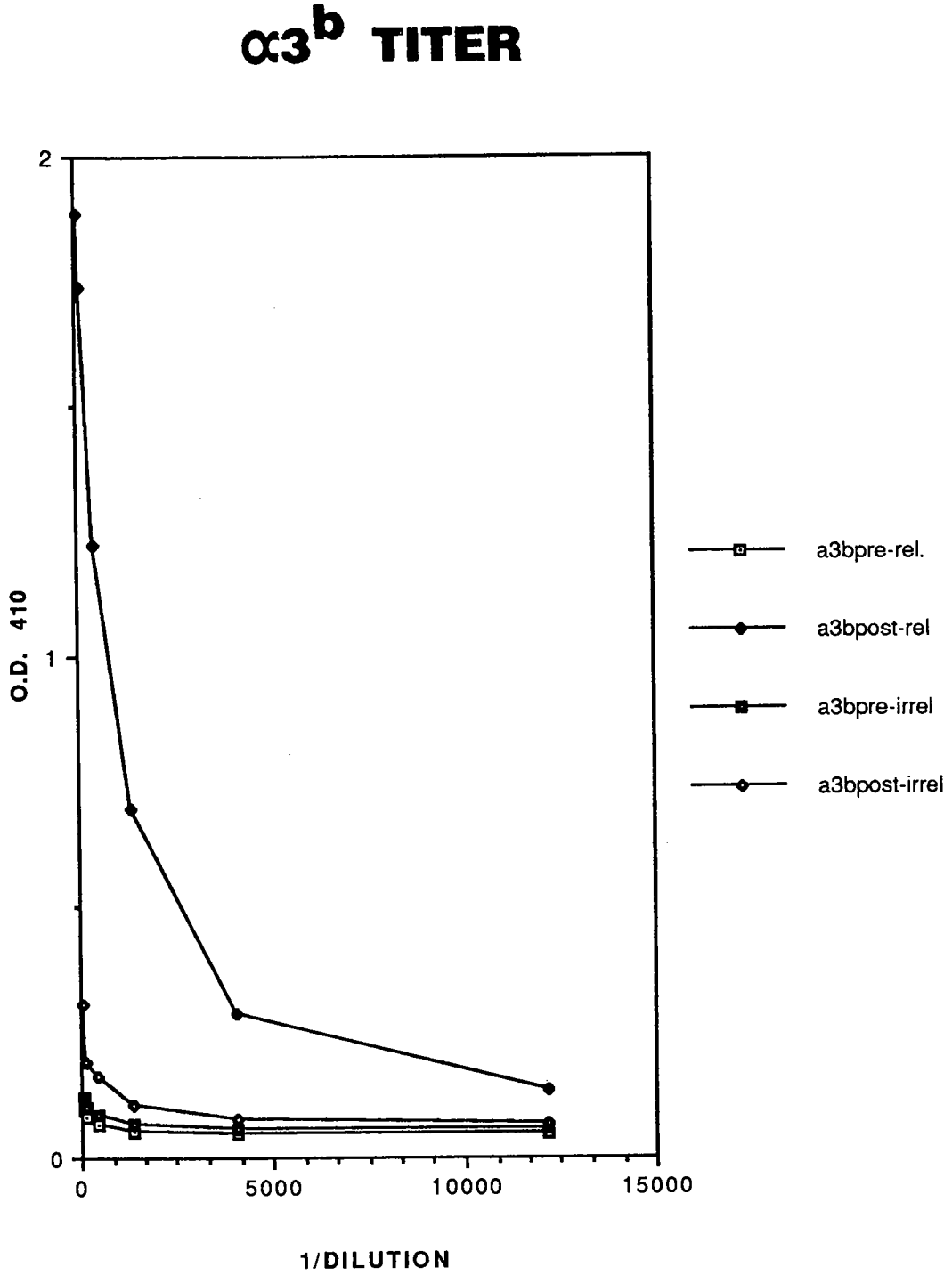


Fig. 3.D.11. ELISA titer of antiserum $\alpha 3^b$, with preimmunization and postimmunization sera, against the respective and an irrelevant peptide.

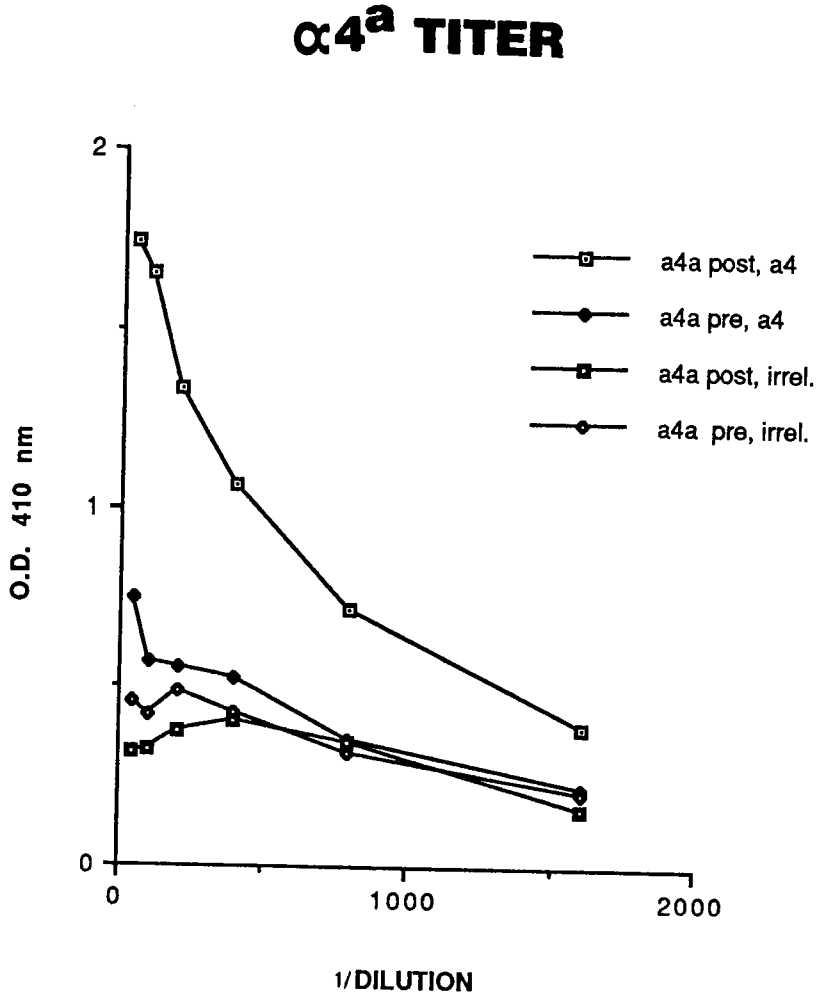


Fig. 3.D.12. ELISA titer of antiserum $\alpha 4^a$, with preimmunization and postimmunization sera, against the respective and an irrelevant peptide.

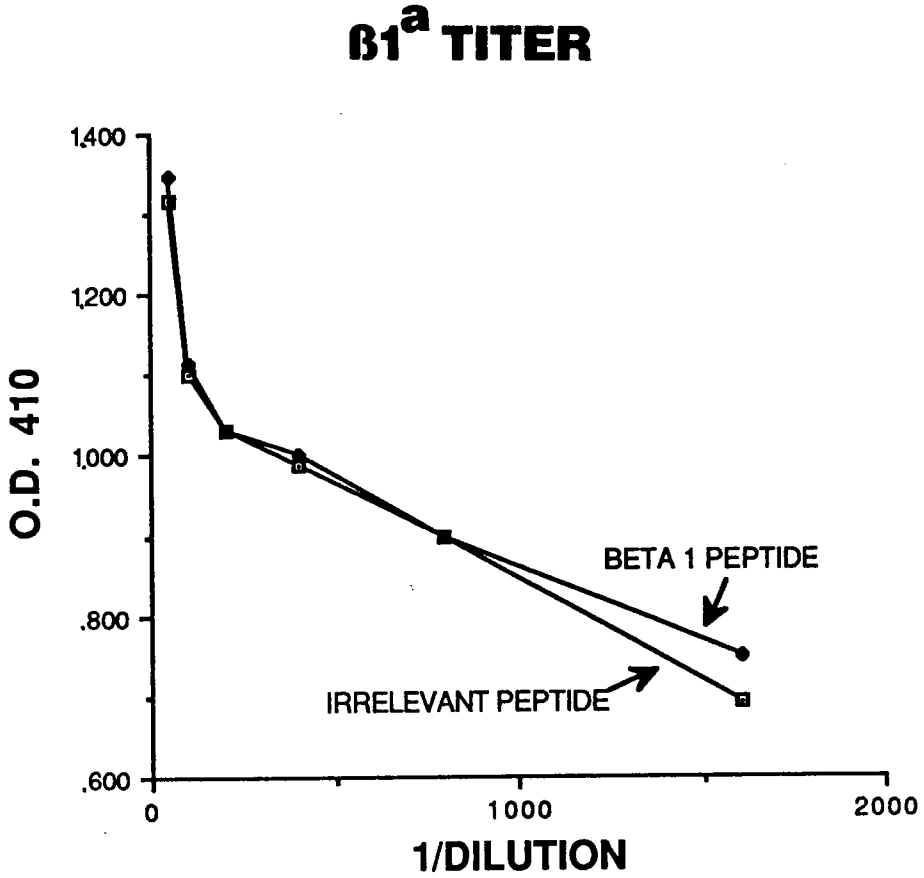


Fig. 3.D.13. ELISA titer of antiserum $\beta 1^a$, with preimmunization and postimmunization sera, against the respective and an irrelevant peptide.

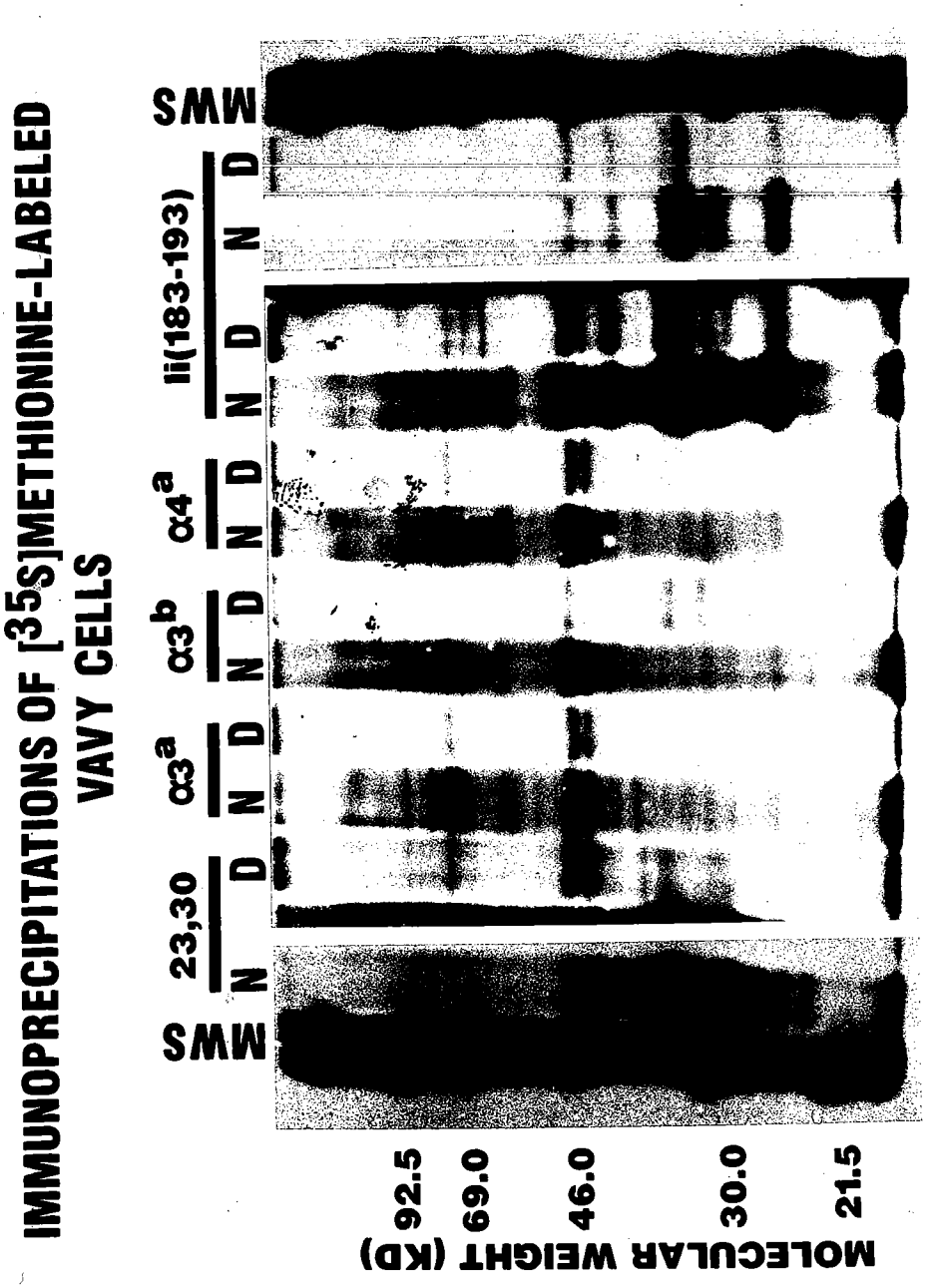


Fig. 3.D.14. Immunoprecipitates of native and denatured Vavy cell membranes, showing that the $\alpha 3^b$ antiserum immunoprecipitates 35 and 32 kD species from denatured membranes. The 35 kD species is probably the alpha chain while the 32 kD species is probably I_i or an unprocessed form of the alpha chain. Panels on either end are films that were exposed to the gel for shorter periods of time.

WESTERN BLOTTING OF WHOLE CELL MEMBRANES

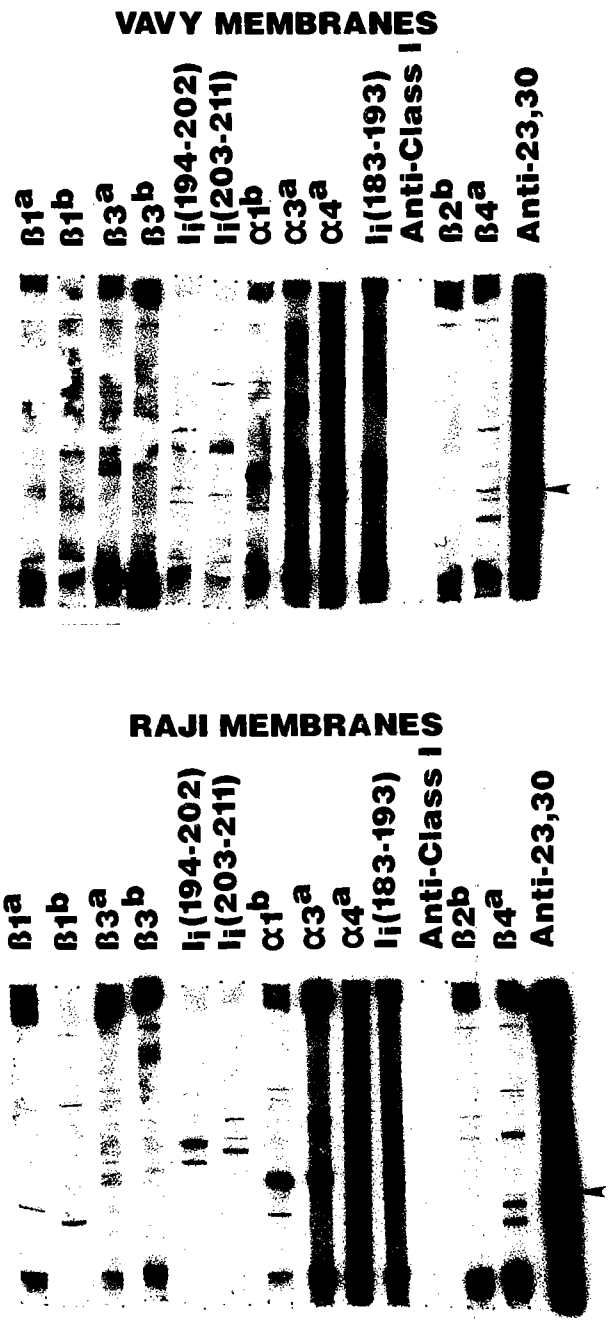


Fig. 3.D.15. Western blot of Raji and Vavy whole cell membrane proteins. Anti-23,30 (anti-class II MHC) recognizes beta chain (arrowhead). $\alpha 3^a$ serum recognizes a band migrating at about 35 kD, probably the class II MHC alpha chain.

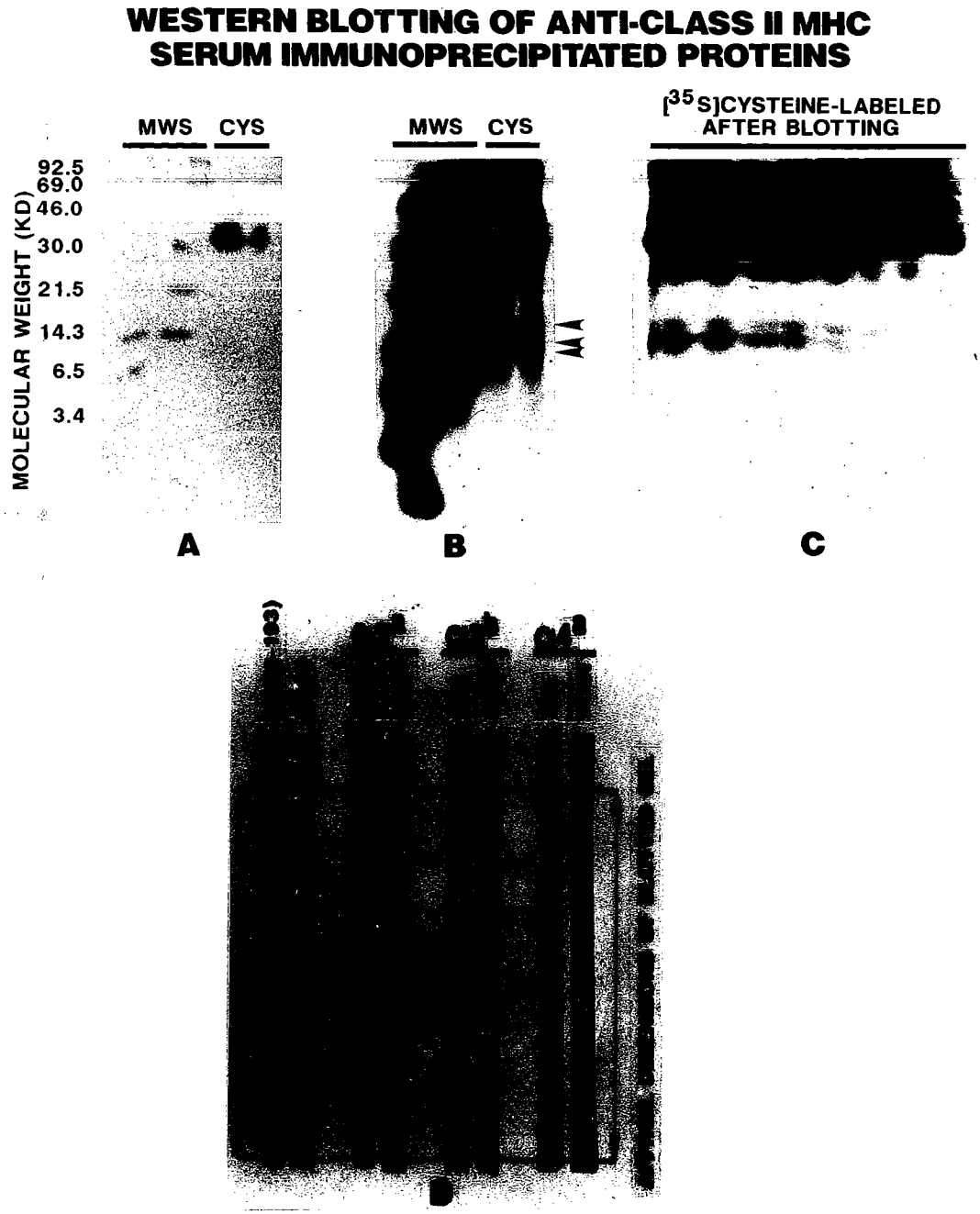


Fig. 3.D.16. Western blot of anti-class II MHC serum immunoprecipitated proteins, detected with goat anti-rabbit antibodies coupled to horseradish peroxidase. Panel A (short exposure) and Panel B (long exposure) show polypeptides at about 15.5, 13.5, and probably one at about 12.0 kD. [^{35}S]cysteine-labeled immunoprecipitated proteins were blotted with various antisera (Panel D), and then autoradiographed (Panel C). $\alpha 3^a$ and $\alpha 3^b$ recognized precipitated class II MHC alpha chains, and $\alpha 3^b$ also recognized a polypeptide in the range of 12 to 16 kD.

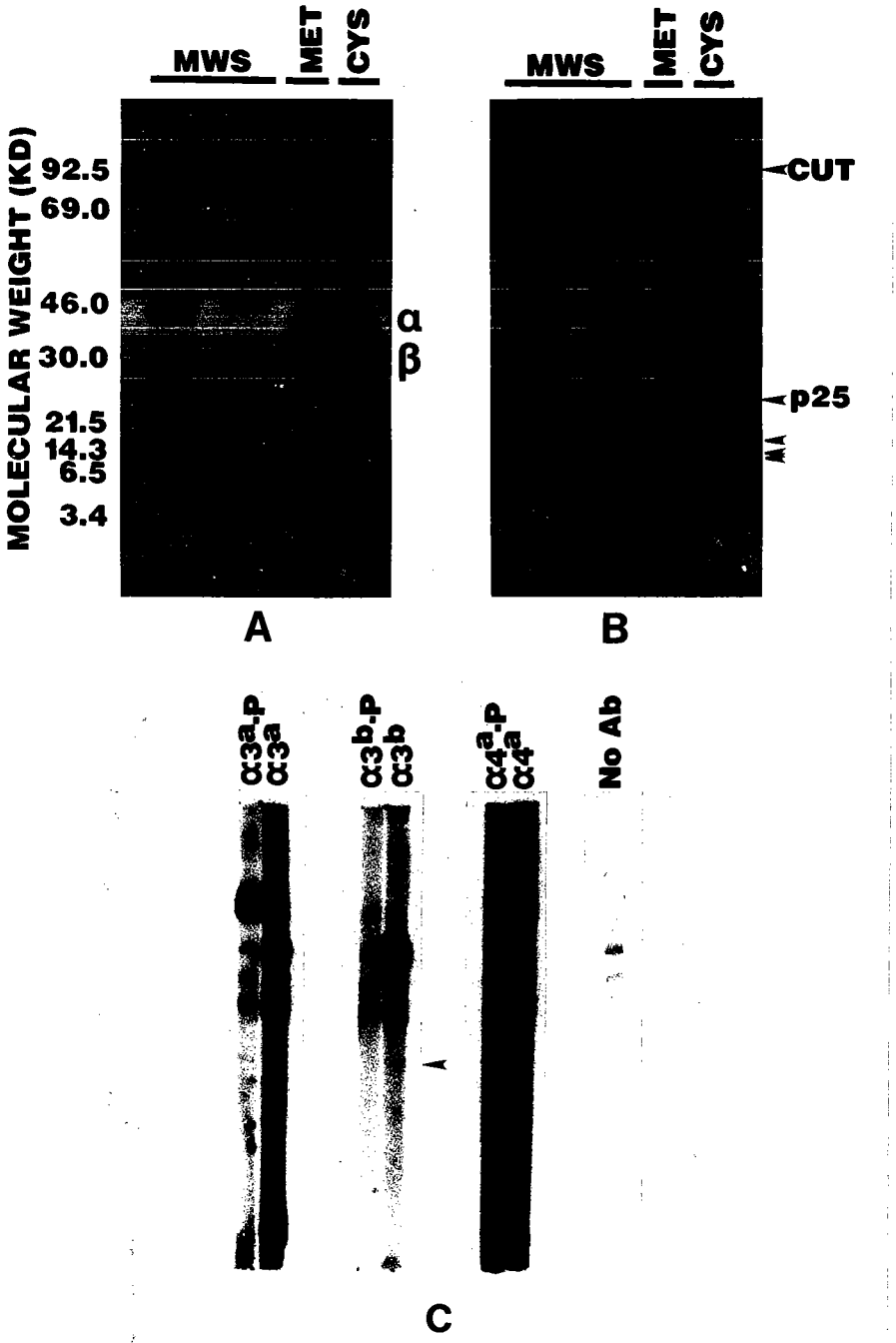


Fig. 3.D.17. Western blot of anti-class II MHC serum immunoprecipitated proteins, detected with [^{125}I]protein A. The [^{35}S]methionine-labeled immunoprecipitate contained alpha chain, beta chain, p25, and a polypeptide about 12.5 kD. The [^{35}S]cysteine-labeled immunoprecipitate contained alpha chain beta chain, and 3 polypeptides about 15.5, 12.5, and 10 kD (arrowheads; Panel A, short exposure; Panel B, long exposure). The [^{35}S]cysteine-labeled proteins were blotted and probed with various antisera (Panel C). $\alpha 3^a$ and $\alpha 3^b$ sera recognize class II MHC alpha chain, and $\alpha 3^b$ serum recognizes a polypeptide about 12.5 kD (all strips exposed for the same period of time).

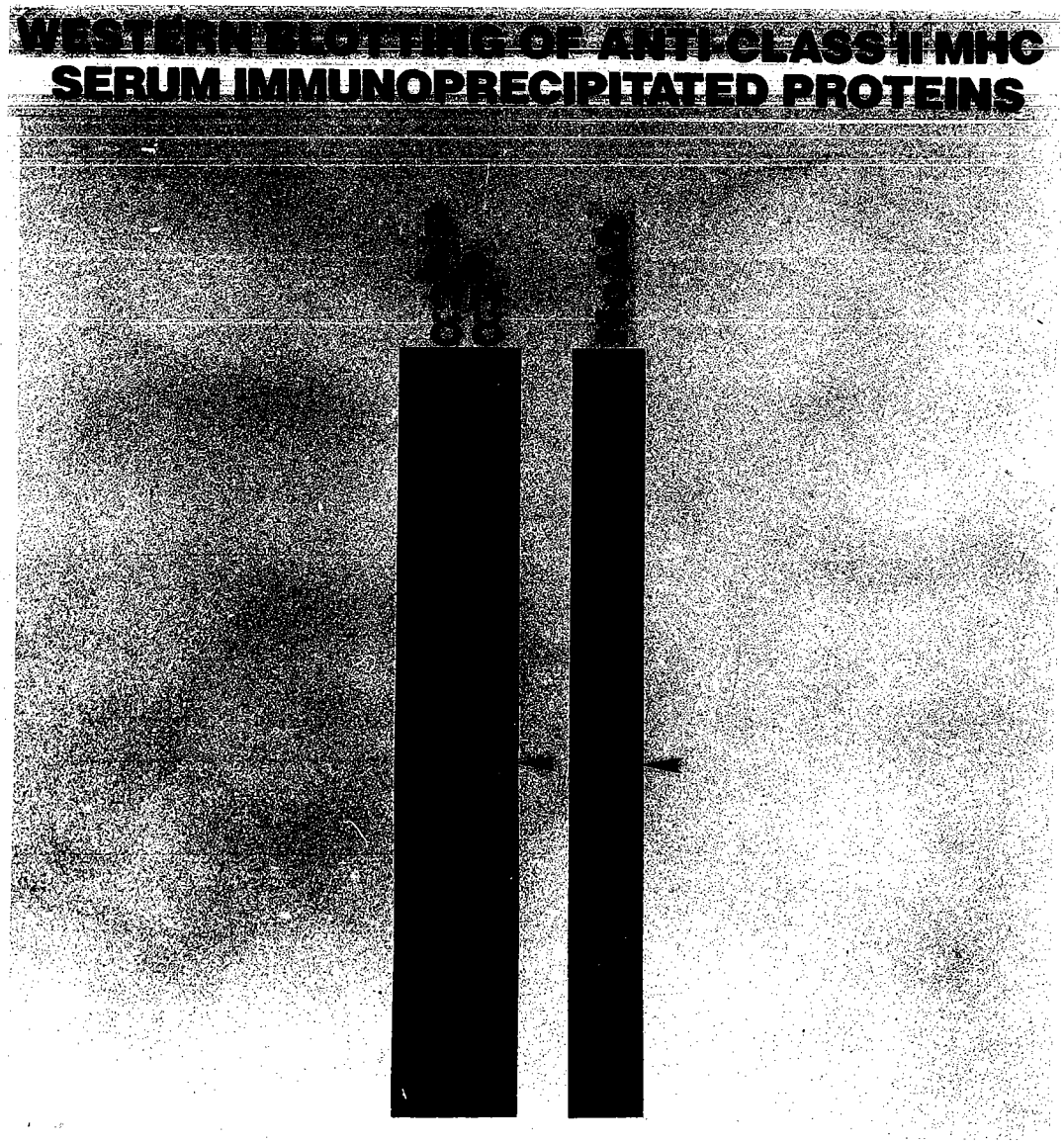


Fig. 3.D.18. Detail and enhancement of Fig. 3.D.17. The polypeptide recognized by $\alpha 3^b$ serum comigrated with an anti-class II MHC serum immunoprecipitated polypeptide. The "No Ab" strip was exposed to the film about 3 times longer than the $\alpha 3^b$ probed strip.

E. Phosphorylation of I_i and the Class II MHC Alpha, Beta and Chains

Introduction. Phosphorylation is a well recognized mechanism for the regulation of the function of receptor proteins. Many systems demonstrating this fact have been worked out in fine detail. The class II MHC alpha chain was demonstrated to be phosphorylated in a B lymphoblastoid cell line (Kaufman *et al.*, 1979). However this report was incomplete, since only a low resolution one dimensional gel was published, and no further information on the phosphorylation reported. Specific goals were the following:

(1) To demonstrate if the class II MHC alpha, beta and the various forms of I_i were phosphorylated, and on which amino acid(s), in B lymphoblastoid cell lines and activated human splenic B cells.

(2) To determine the functional significance of these phosphorylations.

Demonstration of the phosphorylation of I_i and the alpha, beta chains. Raji cells were labeled with [^{32}P]orthophosphate, microsomal membranes were isolated and immunoprecipitated with anti-class II MHC antiserum (Humphreys *et al.*, 1976). Figure 3.E.1, upper panel, shows that both the alpha and beta chains have incorporated radiophosphate. The lower panel of this figure showed that an alternative result of this same protocol could be obtained, I_i alone could be phosphorylated. This problem of disparate results from one

experimental procedure was addressed in further experiments.

Phosphorylation of alpha and beta chains in *S. aureus*-activated human B cells. To address the hypothesis that the phosphorylation patterns seen are a result of the Raji cells being a cell line, further experiments with cells better resembling the *in vivo* situation, *S. aureus*-activated human B cells were done. Small, resting polyclonal human B cells were activated with formalinized, sterilized *S. aureus* for three days, and labeled with [^{32}P]orthophosphate in a manner identical to that in the above section. Again a discrepancy in the results were seen. Phosphorylation of the class II MHC alpha and beta chains were seen as was shown in Fig. 3.E.1, upper panel, and at another time phosphate was incorporated only into I_i. The amount of ^{32}P incorporated in I_i was again low.

Cell density and phosphorylation. To address the hypothesis that the differing phosphorylation patterns resulted from different cell densities at the time of cell harvesting, Raji cells were collected at the following cell densities: 0.33, 0.68, 1.0 and 1.7x10^6 cells/ml. They were labeled and immunoprecipitated by the above protocol and all showed phosphorylated I_i, similar to what is shown in Fig. 3.E.1, lower panel. The amount of ^{32}P incorporated was again low.

Assessment of experimental problems. The problems of non-reproducible results and low incorporation of [^{32}P] forced me to discontinue further experiments on this project. However, the observation of phosphorylation of I_i, alpha and beta chains is valid. To summarize, under seemingly the same conditions, sometimes phosphorylation of the alpha and beta chains would be seen but not I_i, while other experiments would show I_i phosphorylated but not the alpha and beta chains. Further, incorporation in most experiments was so low as to make further protocol modifications (as phosphoamino acid analysis) difficult. The current hypothesis to explain these results was that phosphorylation was dependent on the antigen processing and presentation state of the cells. This would explain the strange results seen in cell lines not presenting antigen, or polyclonally activated cell lines. The observation of the phosphorylation pattern being an either/or situation would indicate that somehow these cells are synchronous with respect to the parameter controlled this effect. Since we did not have a clonal human B and T cell, antigen presenting system this hypothesis was not directly testable. Consequently, it was decided not to pursue the project further. Two other labs had been working on the phosphorylation of I_i and the class II MHC molecules and at the time we was doing this work, early in the course of my thesis work. They reported having similar problems (R. Spiro and V. Quaranta, personal communication).

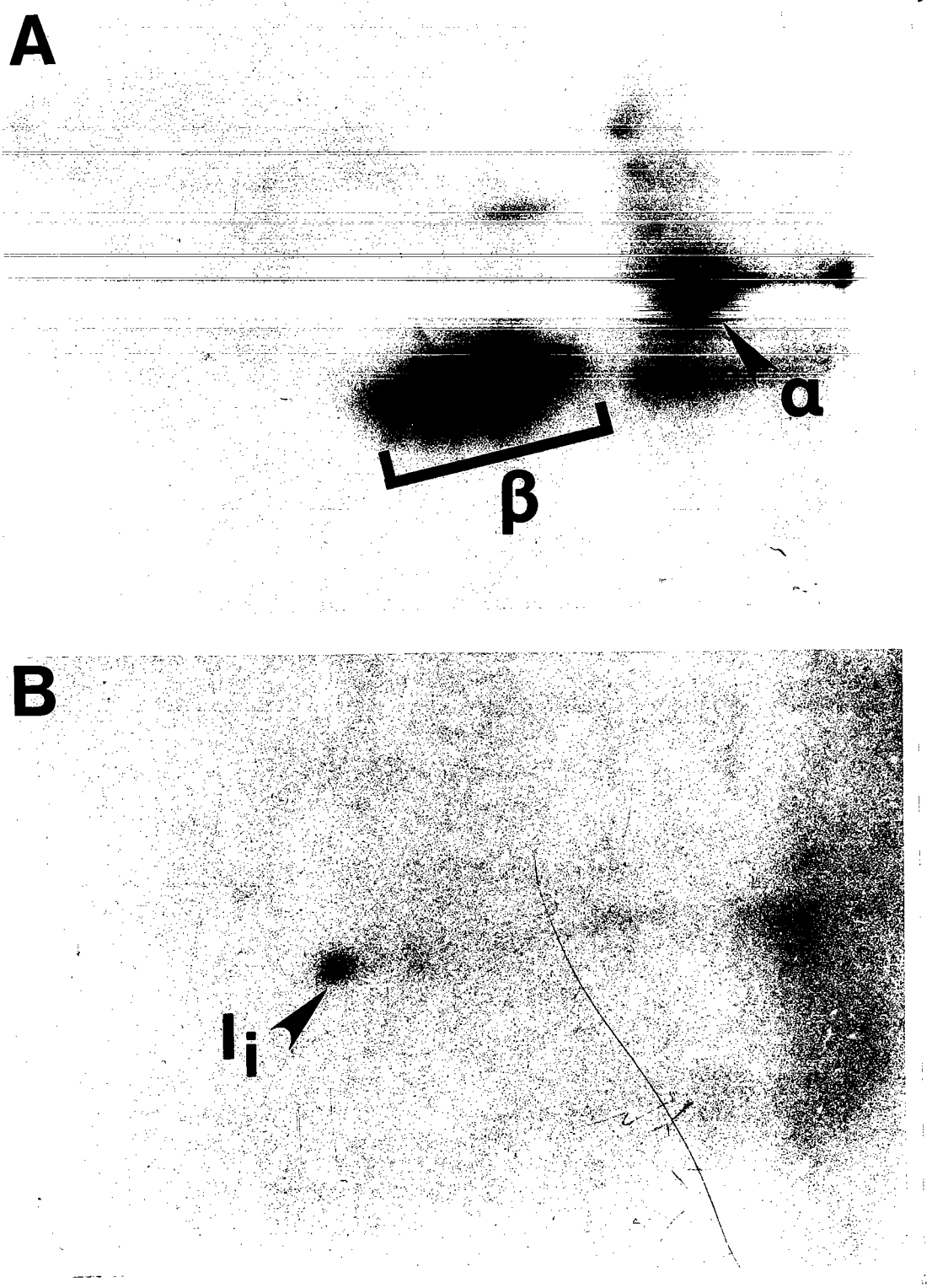


Fig. 3.E.1. Phosphorylation of I_i and the class II MHC alpha and beta chains. Two dimensional NEPHGE and SDS gel of solubilized membranes from [^{32}P]-labeled Raji cells immunoprecipitated with anti-class II MHC antiserum. Panel A shows the alpha and beta chains with incorporated radiophosphate, while Panel B shows I_i with incorporated radiophosphate.

CHAPTER IV

DISCUSSION

A. Structural Analysis of I_i Leading to a Hypothesis on the Binding of an Amphipathic, Alpha Helical Sequence in I_i to the Desetope of Class II Antigens.

Amphipathic helical structure of T cell-presented peptides. An analysis of the strengths and frequencies of periodic hydrophobicities in sequences of well studied protein antigens led to the view that foreign peptides, which are presented to T cells via class II MHC antigens, can be amphipathic alpha helices, *i.e.*, with opposing hydrophobic and hydrophilic sides (DeLisi and Berzofsky, 1985).

Observation of an amphipathic helical region in I_i. Inspection of the amino acid sequence of I_i revealed a striking amphipathic helix running from Glu_{148} to His_{170}. Various graphical and structural models showed that this 5-cycle helix possessed: (1) a narrow strip of aliphatic amino acids (Leu_{150}, Leu_{153}, Met_{157}, Ile_{160}, Val_{164}) down one side of the cylinder, and (2) otherwise, various generally hydrophilic amino acids.

Hypothesis that I_i amphipathic helix protects the class II MHC desetope. This observation led to the hypothesis that this amphipathic helix of I_i fills the antigen-combining site (desetope) of class II MHC antigens until such complexes reach the endosome where dissociation is induced, *e.g.*, by proteolytic cleavage or pH effects.

After such endosomal release, one might fear the "prototypic amphipathic helix" from I_i could compete with foreign peptides for binding to class II MHC antigens. Inspection of structural models, however, led to the hypothesis that the cleaved I_i peptides polymerize tightly, probably as tetramers. Hydrophobic groups along one side of each peptide cylinder interdigitate. Looking at a putative tetramer in which the N-termini of the 4 helical peptides are at one end and the hydrophilic strips meet at the core of the tetramer, side chains of one peptide, Leu_{150}, Met_{157}, and Val_{164}, fit between Phe_{146}, Leu_{153}, Ile_{160}, and Ser_{167} of the clockwise-adjacent peptide (repeating a zipper-like pattern four times). Also, on the external surface of the tetramer, His_{152} and Lys_{163} from one peptide form ionic bridges to Lys_{154} and Asp_{161}, respectively, of the clockwise-adjacent peptide, again as viewed from the N-termini. Finally, at the C-termini, Trp_{168} and Phe_{165} of the adjacent peptides pair hydrophobically. Such polymerization may not occur with the native molecule due to steric inhibition by other parts of the I_i molecule. One can expect that such dimeric or tetrameric polymerization could eliminate I_i peptide competition for the class II MHC antigen desetope, although some other biological activity of

such polymers could remain.

While supported by a structural analysis of the I_i peptide Phe_{146} to Met_{169}, this view has remained only a working hypothesis, that the amphipathic alpha helix Glu_{148} to His_{170} binds to the foreign antigen-binding site of the class II MHC proteins until release, when it polymerizes tightly, no longer to compete with foreign peptides for that site. Although the amphipathic helix, I_i(146-170), has many structural peculiarities favorable to this hypothesis, it could still be only a structural helix of I_i with the hydrophobic strip turned inward to some other relatively hydrophobic region of I_i. In order to test this hypothesis, others in the laboratory have pursued a number of pathways. One set of experiments have attempted to chemically crosslink I_i to the class II molecules. The crosslinked polypeptides have been examined to determine the "nearest neighbor" regions of the three chains.

If this hypothesis is true then it could serve as an explanation for the hyperexpression of I_i in some leukemic cells (Spiro *et al.*, 1980). In such cells the abundance of I_i could alter the capacity of those cells to bind and present antigen. The net result might be a decreased ability for the host's immune system to mount a response against the neoplastic cells, which may be unable to present novel oncogenic antigens.

If the I_i helix does lie in the class II MHC desetope, one might hypothesize this structure is a peptide with near maximal affinity for the desetope. Consequently portions of other proteins that would resemble this region of I_i would have a high affinity for

the desetope, and might be class II presented. Computer programs to search for portions of proteins resembling the structure of this region of I_i were produced, and have since been refined (Reyes *et al.*, 1988; Reyes *et al.*, manuscript in preparation). This method was called the Strip-of-helix algorithm and is covered in detail in the next section.

B. Hydrophobic Strip-of-helix Algorithm for Selection of T Cell-presented Peptides.

The strip-of-helix algorithm. The strip-of-helix hydrophobicity index was the mean hydrophobicity (from Kyte-Doolittle values) of sets of amino acids in axial strips down sides of helices for 3 to 6 turns, at positions n, n+4, n+7, n+11, n+14, and n+18. Our computerized protocol: (1) calculated strip-of-helix hydrophobicity values for 3, 4, 5 and 6 turns of a putative helix (8, 12, 15 and 19 amino acids in length), (2) printed the sequence position, amino acid and index value in tabular form, and index value and sequence position graphically, (3) listed the 5 most highly ranked peptides, (4) indicated those which did not have at least one adjacent strip (±1 or 2 amino acids from position n with hydrophobicity score -1.0 or less, (5) indicated those peptides which contained a proline and (6) indicated peptides which contained a lysine +1 or +3 positions after the C-terminal hydrophobic residue. The peptides correlating well with published T cell responsiveness had: (1) 12 to 19 amino acids (cycles 3 to 5 or 4-6 turns of an alpha helix, where a cycle is a turn plus one amino acid), (2) a strip with highly hydrophobic residues, (3) adjacent, moderately hydrophilic strips, and (4) no prolines. The degree of hydrophilicity of the hydrophilic strips of a putative antigenic helix above a threshold value did not count in the ranking. That is, the magnitude of amphipathicity was not judged to be the principal selecting factor for T cell-presented peptides. This simple algorithm

to quantitate strip-of-helix hydrophobicity in a putative amphipathic alpha helix, allowing otherwise generally hydrophilic residues, predicted 10 of 12 T cell-presented peptides in 7 well-studied proteins (Reyes *et al.*, manuscript in preparation).

Evaluation of method. The strip-of-helix hydrophobicity algorithm well predicted those peptides which experimentally had been found to be T cell-presented (Reyes *et al.*, manuscript in preparation). All except one of the sequences predicted by the algorithm of DeLisi and Berzofsky (1985) were identified with this algorithm. It also scored two antigenic sequences, in beef cytochrome C and in foot and mouth disease virus protein VP1, for which there were no reports of antigenicity. The T cell antigenicity of these sequences could be tested experimentally. Our simpler algorithm consequently performs as well as the DeLisi and Berzofsky method, in this regard.

One advantage of this algorithm is that potentially antigenic regions can be ranked according to their strip-of-helix hydrophobicity indices. With complex or relatively unstudied proteins, one might wish to synthesize and test the highest ranking peptides first. We cannot assess now how far down a series of ranked peptides one might actually find T cell responsiveness.

Some observations are consistent with the view that strip-of-helix hydrophobicity by itself was more important than the magnitude of amphipathicity in predicting antigenicity of peptides. For the hen ovalbumin antigenic peptide Ile_{323} - Ala_{337}, which had

a strip-of-helix hydrophobicity index of +3.36 for 5 turns, an adjacent strip was quite hydrophilic (-2.22 for the strip following Ser_{324}). Another peptide in hen ovalbumin, Ala_{23}-Ala_{37}, which had not been found to be antigenic, had a strip hydrophobicity of +3.02 for 5 turns, and an adjacent strip with a hydrophilicity of -1.40 (from His_{22}). Thus, if some "maximal amphipathic moment" were necessary for antigenicity, it might be somewhere between 5.58 (=+3.36-(-2.22)) and 4.42 (= +3.02-(-1.40)). However, other antigenic peptides examined in hen egg lysozyme and pig proinsulin had much smaller "moments", as calculated in that manner. Hen egg lysozyme peptide Ser_{81}-Ala_{95} had a moment of 3.20 (+1.78 hydrophobicity and -1.42 adjacent hydrophilicity), and pig proinsulin Cys_{6}-Cys_{20} had a moment of 3.70 (+1.96 hydrophobicity and -1.74 adjacent hydrophilicity). Thus, the factors governing antigenicity appeared to be (1) hydrophobicity of one strip-of-helix being among the highest in a protein, if not the highest, and (2) the hydrophobic strip being flanked by one or more moderately hydrophilic strips.

Prediction of the structure of the desetope. The close fit of predictions by this algorithm to experimental findings leads to a structural model of the class II MHC antigen-binding site. Since sequences of computer-selected peptides overlapped those of reported antigenic peptides usually by ten or more amino acids, it would be suspected that if T cell-recognized peptides coil as amphipathic alpha helices, then such peptides would compose helices of at least 4 1/2

complete turns (from amino acid n to n+12). The corresponding T cell receptor could be hypothesized to hold a linear crevice at least 3 helical cycles in length (about 15 Å) to receive such a peptide. In fact, if the I_i amphipathic helix Leu_{150} - Val_{164} fits such a crevice in the class II MHC molecules, the length of that site could be 5 helical cycles or about 27 Å. Loss of the I_i helix might catalyze charging of the crevice with a foreign peptide with a similar hydrophobic strip-of-helix, if removal and insertion were to occur by a concerted mechanism.

Although binding of a peptide's hydrophobic strip of amino acids to class II MHC could be a minimal requirement for its T cell antigenicity, additional structural determinants seem to govern binding and presentation by any given class II antigen allele. Such effects have been documented at both functional and biochemical levels. In studies of I-A^k-restricted T cell clones responding to hen egg lysozyme, Allen *et al.* (1985) found two peptides, Phe_{34}-Arg_{45} and Asn_{46}-Arg_{61}, to account for recognition by 8 of 10 clones. In additional studies of fragments of hen egg lysozyme peptide CM-T11, Shastri *et al.*, (1986) found that peptides Asn_{74}-Ser_{86} and Ser_{85}-Lys_{96} were restricted by I-A^k and I-E^k, respectively. Hen egg lysozyme peptide has been found to bind to purified I-A^k but not I-A^d molecules (Babbitt *et al.*, 1985). In additional studies, hen egg ovalbumin peptide Ile_{323}-Arg_{339} was found to bind to I-A^d but not to I-E^d, I-A^k, or I-E^k (Buss *et al.*, 1986a). Similarly, an alpha helical region of sperm whale myoglobin (Phe_{106}-Arg_{118}) has been shown to be presented by I-A^d

(Cease *et al.*, 1986) and not by I-E^d or some other class II MHC alleles (Berkower *et al.*, 1984; Berkower *et al.*, 1985). It is likely that structures on alpha helical peptides, in addition to the axial strip of aliphatic residues, govern the binding to specific alleles of class II MHC molecules.

Since the original publication of this algorithm (Elliott *et al.*, 1987; Stille *et al.*, 1987) the structure of the class I MHC molecule has been solved (Bjorkman *et al.*, 1987a; 1987b). By sequence comparison of class II to this structure, a hypothetical model of the class II MHC desetope has been predicted (Brown *et al.*, 1988). That model well supported the above predictions of the class II MHC desetope structure. The desetope is made up of an alpha helix of the alpha chain on one side and the beta chain on the other, each comprised of hypervariable residues. The walls of the desetope extend for 6 or 7 turns of the helix. The size of the desetope cleft might be thinner than the diameter of an alpha helix (T. Garrett, personal communication). This, however, might reflect the lack of a conformational change that occurs when a foreign peptide binds in the desetope.

Prediction of class I MHC-presented peptides. Similar selection of peptides for class I MHC-mediated presentation of antigen to cytotoxic T lymphocytes (CTL) might also be partially governed at a peptide-binding stage. Townsend *et al.* (1986a) has analyzed class I MHC haplotype-specific presentation to CTL of various peptides derived

from influenza virus nucleoprotein. They demonstrated the selective presentation of nucleoprotein peptides Ile_{365}-Glu_{380} and Ser_{335}-Gly_{349} by murine H-2D^{b} and human HLA-B37 class I MHC molecules, respectively. Since influenza virus nucleoprotein is not expressed on the surface of an infected cell (or on the viral envelope) and since anti-hemagglutinin CTL kill cells transfected with leader sequence-free hemagglutinin (which is not surface-expressed as intact hemagglutinin), Townsend *et al.* (1986b) hypothesized that class I-restricted antigens are digested to peptides prior to surface expression of the CTL-recognized fragment. Reyes *et al.* (1988) used the strip-of-helix algorithm to predict class I MHC-presented peptides from four proteins. The selected peptides well matched the portions of the proteins shown experimentally to be class I MHC-presented.

Similarity to peptide hormones. Amphipathicity is a physical characteristic required for the function of many peptide hormones (Kaiser and Kézdy, 1984), as well as for recognition of antigenic fragments (DeLisi and Berzofsky, 1985). One might then question whether strip-of-helix hydrophobicity (and its derivative, amphipathicity) represent an evolutionary convergence of forms to fit a general function, or a divergence of forms from an original function. That is, in early multicellular organisms regulated by amphipathic helical peptide hormones, there might have evolved a need to discriminate self-hormones from structurally similar foreign peptides. Primordial T cell receptor function could have diverged from

those receptors which functioned to recognize such homeostatic peptide hormones, or alternatively the two receptor types may have evolved from a common ancestor, possibly a primordial peptide hormone receptor.

Design of peptides for synthesis. In selecting class II MHC-presented peptides of a protein for synthesis, one might wish to examine strip-of-helix hydrophobicity plots over 3-, 4-, 5-, and 6-turns of an alpha helix. In such plots many peptides which have been proven to be antigenic appeared as third-, fourth- or fifth-ranked positions in a 3- or 4- turn plot, but in a 6-turn plot they occupied first- or second-ranked positions (Table 4.B.1). Many of the experimentally antigenic peptides, thus, showed up consistently in 3- to 6-turn plots. If one does not see a consistent or evolving ranking in a comparative analysis of 3-, 4-, 5-, and 6-turn plots, we would suggest a 5-turn plot to be preferred for ranking putatively antigenic peptides because it tends to rank highly most peptides which are found experimentally to be antigenic.

For the synthesis of a potential immunogen, we would not feel restricted to the peptide bounded by the first and last hydrophobic amino acids in the hydrophobic strip. A proline beyond the N-terminus (plus a further N-terminal amino acid) would create a hairpin turn of the peptidyl backbone in which the carbonyl function of the amino acid preceding the proline could hydrogen bond to the amido function of the second amino acid following the proline (Dyson *et al.*, 1985) to

stabilize the N-terminus of the helix. Consequently these amino acids, if present in the protein sequence, could be included in the synthesized peptide.

Spouge *et al.* (1987) found an increased frequency of lysines in the C-terminal regions of experimentally antigenic peptides. They proposed that lysine could bind to the desetope and/or stabilize the C-terminus of an alpha helix. Alternately, one could suggest that tryptic cleavages, which produced many of the experimentally antigenic peptides, would leave a disproportionate number of C-terminal lysines. However, a closer examination of the sequence position of the lysines showed a preponderance (7/12) of the lysines occurred in the position immediately following the last amino acid in the hydrophobic strip-of-helix ($+100^{o}$ with respect to the axis of the cylinder). 0/12 occurred at the second following position ($+200^{o}$). 2/12 occurred at the third or later positions. This distribution of lysines with respect to the termination of the hydrophobic strip-of-helix, supported the view that a desetope interaction with the lysine occurred, perhaps with a carboxyl function of the desetope, positioned on the side of a trough complementary to the C-terminus and $+100^{o}$ to the last hydrophobic residue in the antigenic peptide's strip-of-helix. Given that empiric observation, one might consider adding a C-terminal lysine in the synthesis of a peptide immunogen (whether or not it occurred in the sequence of the antigenic protein).

One might also wish either to substitute Phe with Tyr, or to add a terminal Tyr to permit radioiodination of the peptide. Additional hydrophilic N- or C-terminal amino acids could be included to improve

peptide solubility. While this computer program specifies explicitly and ranks potentially antigenic sequences, additional considerations could determine the exact peptide for synthesis and further experiments.

Table 4.B.1. Selection of peptides as a function of turns in a putative alpha helix.

	Lysozyme		Ovalbumin		Sperm Whale Myoglobin	
Reported Sequences:	46-61 74-86 81-96 108-119		323-339		68-78 102-118 132-146	
Predicted Sequences:		index		index		index
3 turns						
	120-127	3.73	79-86	4.17	107-114	4.40
	88-95	3.50	83-90	4.03	131-138	2.83
	2-9	2.83	114-121	3.93	21-28	2.77
			245-252	3.93	61-68	2.53
4 turns						
	88-99	3.67	79-90	4.25	131-142	3.25
	98-109	2.55	323-334	3.75	21-32	3.02
	71-82	2.42	364-375	3.40	10-21	2.92
	84-95	2.32	23-34	3.32	61-72	2.85
	25-36	2.25			104-115	2.82
5 turns						
	88-102	2.86	323-337	3.36	61-75	3.18
	84-98	2.76	79-93	3.08	100-114	3.08
	71-85	1.78	23-37	3.02	123-137	2.40
	81-95	1.78			21-35	2.26
					29-43	2.00
6 turns						
	84-102	2.23	323-337	3.36	131-149	2.15
	81-99	2.18	364-375	3.15	57-75	2.15
	91-109	1.87			97-115	2.10
	88-106	1.80			61-79	2.00
	76-94	1.60				

C. Proteolysis of I_i.

Proteolysis of I_i to p25. p25 was shown to be an exomembranal, C-terminal fragment of I_i, by second immunoprecipitations with various antibodies of VIC-Y1 mAb-immunopurified I_i-containing complexes, that were denatured. Rabbit antisera to two C-terminal I_i peptides immunoprecipitated the denatured and resolubilized p25, while an antiserum to an N-terminal peptide failed to precipitate p25. In the same gels, each of these three antisera recognized denatured and resolubilized I_i. A monoclonal antibody to I_i immunoprecipitated [^{35}S]methionine-labeled p25 but not [^{35}S]cysteine-labeled p25, consistent with the loss of a portion of I_i containing the only cysteine in I_i, Cys_{28}. Judging from the 25 kD weight of p25, the lack of reactivity with an antiserum to the N-terminus of I_i, the absence of Cys_{28}, and positioning of the transmembranal domain (Gly_{31}-Tyr_{56}), one can conclude that p25 represented the C-terminal portion of I_i. p25 would then contain the amphipathic, alpha helical region I_i(146-170) which was hypothesized to fit in the class II MHC desetope.

Cleavage of I_i *in vivo* appeared to occur while the molecule was associated with class II MHC molecules, since some p25 was found in immunoprecipitates with an anti-class II MHC heteroantiserum which did not recognize I_i in immunoprecipitates from a class II^-, I_i^+ cell line (Spiro *et al.*, 1985). Since immunoprecipitates with anti-I_i sera demonstrated perhaps 5-fold more p25 than did

precipitates with anti-class II MHC serum, one can suggest that although p25 was cleaved while associated with class II MHC antigens, some p25 might have been generated by proteolytic cleavage of I_i not associated with class II MHC antigens, or had since dissociated from the class II MHC antigens. I_i has been shown to have a transmembranal segment cleavage site which is sensitive to a signal peptidase and can be exposed upon deletion of hydrophilic amino acids on the N-terminal side of the transmembranal segment (Lipp and Dobberstein, 1986).

The relationship of p25 to I_i was also suggested by Giacoletto *et al.* (1986) who demonstrated that I_i and p25 derived from SDS electrophoretic gels of immunoprecipitates had similar tryptic peptide maps.

Proteolytic cleavage of class II MHC antigen-associated I_i to p25 appeared to be determined by secondary structural characteristics which left the most sensitive site for cleavage to p25 external to the transmembranal segment. Since cleavage with three proteases with different specificities all yielded initially fragments about 25 kD, one can suggest that *in vivo* cleavage of I_i to p25 is restricted by secondary structural features (domains), as with IgG (Porter 1959), rather than being the result of an exquisite substrate specificity of some endogenous protease. Marks and Cresswell (1986) digested class II MHC antigen-I_i complexes with proteinase K and identified in immunoprecipitates the principal I_i fragments to be from the C-terminal portion of I_i and to be associated with class II MHC antigens. They did not address the origin of *in vivo* generated p25,

but their data are consistent with the hypothesis that such a C-terminal fragment of I_i can remain associated with class II MHC antigens after proteolytic cleavage at a site near its transmembranal region. Although alternate translational start sites and exon splicing accounted for the 4 forms: I_i, $\gamma 2/\gamma 3$, p41 and p43, no genomic basis for a p25-coding transcript has been found (Strubin *et al.*, 1986b).

Cleavage of I_i to p25 occurred in an ER or cis-Golgi compartment. Since p25 was completely sensitive to treatments with endoglycosidases F and H, its carbohydrate side chains were not processed to complex sugar forms. It was therefore considered to be derived from a high mannose form of I_i. Also, since anti-I_i(183-193) serum, which immunoprecipitates p25, did not recognize either O-linked forms of Ip, or the I_i-CS proteoglycan form of I_i, p25 was probably not derived from either of those molecules.

Immunoprecipitation of Percoll-density-gradient fractions from Dounce-homogenized, polyclonally activated B lymphocytes, which were [^{35}S]methionine, pulse-chase-labeled, demonstrated the appearance of p25 at 20-40 min chase times in Golgi-ER fractions, and not in either plasma membrane or lysosome fractions (Nguyen *et al.*, 1988). The placement of the I_i cleavage to p25 in the ER or cis-Golgi, during this time frame, is also supported by work with vesicular stomatitis virus which showed that some proteins transit through the Golgi between 13 and 60 minutes (Morrison and Ward, 1984).

The appearance of p25 in an ER or cis-Golgi compartment is consistent with the hypothetical functions: (1) that there could exist an ER degradative pathway to destroy incomplete complexes of class II

MHC proteins and associated molecules, or (2) that I_i could retard the release of class II MHC molecules to the cell surface until they have bound foreign peptide. Such foreign antigen-containing complexes could be carried to the cell surface by bulk flow. Precedent for the former hypothesis includes the description of a pre-Golgi proteolytic pathway for rapid degradation of newly synthesized, T cell receptor subunits (Lippincott-Schwartz *et al.*, 1988). This pathway appears to serve in the degradation of "unassembled" or "incompletely assembled" T cell receptor α, β, δ proteins, and is not sensitive to inhibition with lysosomotropic agents such as ammonium chloride, chloroquine, and methylamine. This pathway is the sole destructive mechanism followed by T cell receptor α chains and α-β complexes in transfected fibroblasts. The degradation of I_i to p25 has the characteristics of this ER degradative pathway, including: (1) the relative insensitivity to treatments with chloroquine or monensin (Nguyen *et al.*, 1988), (2) time of cleavage of about 20 min, and (3) cleavage of high mannose species consistent with the T cell receptor complex cleavage in an early compartment. Precedent for the latter model can be found in the work on the p78 BiP protein which associates with mIgM heavy chain until release of IgM to the surface (Bole *et al.*, 1986; Hendershot and Kearney, 1988).

Consequently it was concluded that the cleavage of I_i to p25 does not occur in a post-Golgi compartment, for example, upon fusion with an endosomal vesicle with digested foreign antigen. This degradation to p25, however, could still be associated with a regulatory event of class II MHC antigen-charging with foreign

peptides. Fusion of endosomes with ER and cis-Golgi compartments has been described (Opresko and Karpf, 1987). It is also possible this cleavage could reflect destruction of I_i molecules which are synthesized in excess of the number required to associate with all nascent class II MHC alpha-beta chain complexes. However, since I_i is processed to complex sugar and O-glycosylated forms, beyond the point at which p25 is generated, additional functions for such mature forms are likely.

Future work of this laboratory will be aimed at defining the cleavage site which generates p25 and to test whether release of I_i or p25 has a catalytic relationship to antigen charging of class II MHC molecules *in vitro* and *in vivo*.

Proteolysis of I_i via a late pathway. Another pathway of I_i proteolysis has been described (Blum and Cresswell, 1988; Nguyen *et al.*, 1988). Incubation of cells with leupeptin or antipain, but not with chymostatin or pepstatin, revealed proteolytic intermediates p21 and p10, at 2-5 hr after synthesis of the class II MHC-I_i complex. These two species were determined to be derived from the N-terminal region of I_i (Fig. 4.C.1) by peptide mapping and precipitation with anti-I_i peptide antibodies of denatured molecules. Blum and Cresswell (1988) called this the "LIP" pathway, for Leupeptin-Induced Proteins. Since p21 was immunoprecipitated with anti-class II MHC antibodies which did not recognize I_i (Spiro *et al.*, 1985), at least the initial step in the path of I_i proteolysis occurred while

I_i still adhered to the alpha and beta class II MHC glycoproteins. Furthermore, digestions with Endo H and Endo F (Blum and Cresswell, 1988) and the sialic acid-induced electrophoretic heterogeneity (Nguyen *et al.*, 1988) of p21 indicated it was derived from a form of I_i that had N-linked sugars of the complex form. These observations led to the view that the cleavage of I_i to p21 and p10 occurred in a post-Golgi or endosomal compartment. As further confirmation for this site of cleavage, cells which were incubated with monensin, trapping I_i in the Golgi, produced no p21 or p10. In pulse-chase experiments, these polypeptides were seen to be produced maximally about 2-5 hr after synthesis, as would be consistent for a site of cleavage in a post-Golgi or endosomal compartment.

The conclusion is that a late form of I_i was degraded by a leupeptin insensitive enzyme to the products p21 and p10. Normally these species were quickly degraded by a leupeptin sensitive enzyme to smaller products, that would not have been seen in experiments using routine protocols. This would be consistent with the removal of I_i from the class II MHC molecules during antigen presentation. Possibly p21 and p10 are the remnants of I_i after removal from the desetope.

Comparison of the pathways of degradation of I_i. Two distinct pathways of intracellular proteolysis of I_i have been described. In the first pathway, an early, high mannose form of I_i was degraded to p25 (the C-terminal portion of the molecule). This cleavage probably took place in the ER or cis-Golgi compartment about 20 min after

synthesis. Its function could be as a pathway for the degradation of incompletely formed complexes of class II MHC molecules and associated proteins, as has been described for the T cell receptor (Lippincott-Schwartz *et al.*, 1988).

In contrast, cleavage of I_i via a later pathway resulted in two products, p21 and p10, that were derived from the N-terminus of the I_i molecule. This cleavage took place about 2-5 hr after synthesis, probably in a post-Golgi or endosomal compartment. This pathway might be the more immunologically relevant pathway, with p21 and p10 possibly being the remnants of I_i cleaved away from the class II MHC molecules as foreign antigen combined, as the class II MHC molecules were being prepared for presentation on the cell surface.

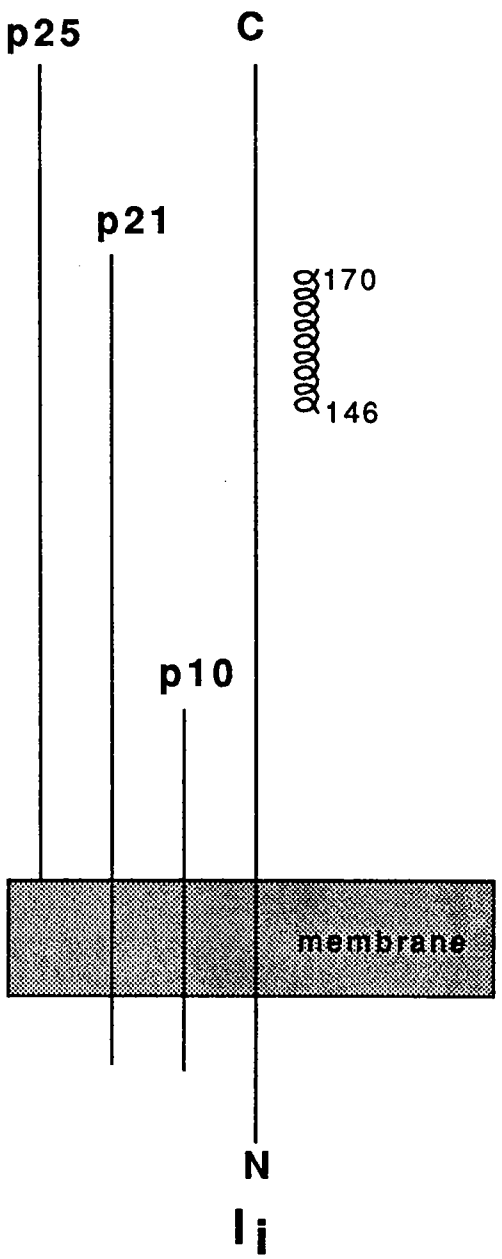


Fig. 4.C.1. Identity of p25, p21, p10. p25 contains the C-terminal portion of I_i, while p21 and p10 contain more of the N-terminal region. The position of the I_i amphipathic alpha helix (146-170) is indicated.

D. Proteolysis of the HLA Class II MHC Alpha and Beta Chains.

Putative sites for regulatory proteolytic cleavages. We searched class II MHC alpha and beta chains for conserved, basic amino acid pairs, which in other systems are known to be sensitive targets for physiologically regulatory, proteolytic cleavages, *e.g.*, in proinsulin (Steiner *et al.*, 1969) and the hemagglutinin of influenza virus (Skehel and Waterfield, 1975). In class II MHC antigen alpha chains, we found Arg_{42} or Lys_{42}-Lys_{43}, Lys_{79}-Arg_{80} (DR and DQ), Arg_{150}-Lys_{151} (DR only), and Arg_{222}-Lys_{223} (DR only). In beta chains we found Lys_{71}-Arg_{72} (in most DR and DQ), and Arg_{93}-Arg_{94}, which is particularly well conserved.

When the positions of these sequences were examined in relation to the predicted structure of the class II MHC molecules (Brown *et al.*, 1988) interesting observations were made. In both chains, the walls of the desetope were bracketed by, or contained, potential cleavage sites. It was hypothesized that the destruction of the desetope would be one of the most efficient ways to control the antigen presenting function of the class II MHC antigens. These cleavage sites seem to be in crucial positions to do just this. Alternatively, one could hypothesize that after removal of one wall of the desetope that helix could be replaced by a similar structure, in a process of "desetope conversion". Furthermore excised fragments could be presented by other, intact desetopes leading to MHC linkage of autoimmune responses to structurally similar self and foreign

proteins.

Rationalization of putative polypeptides to observed polypeptides. If one assumes complete cleavage at all the proposed sites, nine polypeptides would be produced (Table 4.D.1). Two of these polypeptides might be recognized with the anti-α3 serum (4.0 kD and 7.8 kD). The former polypeptide, α(80-150), contains the site of a complex carbohydrate addition, which could increase the molecular weight of this fragment to about 13.0 kD. This would put it in the range of the observed polypeptides in Fig. 3.D.16 to 3.D.18. If the α(80-150) polypeptide is that band to which $\alpha 3^{b}$ blots, then it will not line up with the methionine labeled band in this range, since the sequence contains no methionines. Consequently future experiments will be aimed at determining with which radiolabeled band the blotted band comigrates. Also immunoprecipitates treated with Endo F should drop the molecular weight of the blotted band to about 7.7 kD. Alternatively, if a polypeptide DRα(43-150) was formed, it would contain both methionines and cysteines, and be recognized by the anti-α3 serum.

It was unfortunate that none of the anti-beta chain rabbits produced specific antibodies. However, the goal of determination of a beta chain origin of some of these polypeptides is still possible. As seen in Table 4.D.1 each polypeptide is unique with respect to amino acid content and carbohydrate. Polypeptide β(1-71) will label only with cysteine and contain carbohydrate, polypeptide β(72-80) will

label with cysteine but be under 1.0 kD and might not be resolved, polypeptide β(81-93) will only partially label with methionine, and probably will be too small to resolve (1.3 kD), and polypeptide β(94-238) will label with both cysteine and methionine, and will migrate at about 16.0 kD. Such examination of the polypeptides is possible but the best plan will be to resynthesize the peptides and produce more antisera with the recommendations mentioned below.

If all of the potential cleavage sites are not used, or if others are used, then the situation would become more complex to interpret. If that were the case, one could still draw conclusions if all data (results from blotting, amino acid incorporation, molecular weight, carbohydrate labeling, etc.) was assembled and systematically compared to the known sequences of the alpha and beta chains.

Possible role of class II MHC proteolytic cleavages: desetope inactivation. One possible function of the proteolytic cleavages of the alpha and beta chains could be in the destruction of the antigen presenting capabilities of the surface-expressed class II MHC molecules and consequent attenuation of antigen presentation to T cells. To address this hypothesis future experiments will involve examination of surface-expressed class II MHC molecules for their pattern of alpha and beta chain cleavages. This experiment will be done using a technique that couples biotin to the cell surface molecules (Jasiewicz *et al.*, 1976; Elliott *et al.*, 1989). These labeled molecules can then be detected using [^{125}I]-avidin. Dr. W.

Elliott, formerly of this laboratory, demonstrated that class II MHC molecules can be labeled well by this technique. Further, pulse-chase experiments in conjunction with the biotinylation could be done to address the question of how long after surface expression are the chains cleaved.

One must rationalize hypotheses about the role of the putative cleavage sites with the observation that some of the pairs of basic residues are not conserved in all isotypes or alleles of the genes. Lack of these putative cleavage sites may be found to be significant in possible isotype-specific functions of the DR, DP, and DQ complexes. Alleles which lack these cleavage sites might be able to be linked to altered antigen presentation functions, or certain diseases.

Possible role of class II MHC proteolytic cleavages: desetope conversion. Another possible function of the proteolytic cleavages of the class II MHC alpha and beta chains could be to aid in antigen presentation by allowing the binding of a peptide similar to one wall of the desetope. Some antigenic peptides have sequence homology to the putatively excised, hypervariable region peptides of the alleles which restrict T cell presentation of the antigenic peptides (Guillet *et al.*, 1987). This finding led Guillet *et al.* (1987) to suggest that these hypervariable regions might be displaced in some fashion by the binding of the foreign peptides, although excision at the bracketing pairs of basic amino acids was not proposed. We would now suggest, specifically, that endosomal proteolytic cleavage of the beta chain at

Lys_{71}-Arg_{72} and Arg_{93}-Arg_{94} or of the alpha chain at Lys_{42}-Lys_{43} and Lys_{79}-Arg_{80} with consequent removal of the cleaved peptides could expose a site which could bind peptides structurally similar to the excised peptide.

Closer examination of this region in the beta chain revealed a conserved sequence Cys_{79}-Arg_{80}-His_{81}-Asn_{82}-Tyr_{83} which was present in all reported DR, DP and DQ alleles (Figueroa and Klein, 1986). While Arg_{93}-Arg_{94} was present in all reported DR, DP and DQ beta chain alleles, Lys_{71} was substituted in some alleles. One might suggest that Arg_{80}-His_{81} represented a cleavage site more readily identified under acidic conditions and that Cys_{79} (site of a disulfide bond) could stabilize the peptide fragment in the N-terminal portion of this hypervariable region. That is, the entire hypervariable region appeared to consist of two subregions with a potential for preferential excision of a 3 1/2 cycle helix (His_{81}-Arg_{93}) under some conditions.

Problems of producing anti-peptide antisera. We had problems producing some anti-peptide antisera. The most likely explanation was that most of the peptides we synthesized were too short. The unsuccessful peptides (β1-4 and α1-2) were between 7 and 9 amino acids long, while the successful peptides were 12 and 13 amino acids long. Many reports seem to favor longer peptides for immunogens (Ziltener *et al.*, 1987) and several rationales support this view. Longer peptides provide a logarithmically increasing number of possible

epitopes, consequently giving a higher probability of activating more clones. Using this logic, the rabbits that were defined as non-producers could actually have responded to the peptide, but only with a few clones. The resulting antibody response might have been too low to detect. Also shorter peptides have a higher probability of having their structure altered by the coupling to the carrier (Briand *et al.*, 1985).

Another explanation of the low success rate is that only two rabbits were used per peptide. It is known that some rabbits do not respond well to some antigens, possibly due to the fact that they are necessarily genetically identical with respect to the MHC. The peptide conjugate could be injected into additional rabbits.

Future experiments. There is much work left to be done on this project. The primary goal is to conclusively define the origin of the polypeptides. To achieve this I would: (1) resynthesize the peptides and repeat the antisera production following the recommendations above, (2) repeat blotting experiments with a urea gel system, or another gel system, that would expand the separation of the peptides allowing identification of which bands comigrate, (3) use a blotting system with luminol, a substance which when used in conjunction with HRP will expose a film with light (Amersham Corp.), which would eliminate background bands due to ^{35}S while allowing overexposure, and (4) repeat blotting experiments after digestion with Endo F and Endo H to determine what peptides contain carbohydrate.

Other experiments that will be important to do will be to (1) follow the production of these peptides with [^{35}S]cysteine pulse-chase experiments to determine when these peptides are produced *in vivo*, (2) surface label the class II MHC molecules with biotin and determine the relationship of surface expression and susceptibility to proteolytic cleavage, and (3) repeat the biotin labeling with pulse-chases to determine how long after surface expression does cleavage take place.

CHAIN	PORTION	Number of Amino Acids (Approx. M.W.)	carbohydrate	Amino Acids Present in Putative Polypeptide (Vavy Haplotype) DR3	DP1	DQ2	Recognized by Anti-Serum
alpha	1-42	42 (4600)		M-27 M-40	M-27 M-35		α1 α2
alpha	43-79	36 (4000)	Asn_{70} High Mannose	M-77			α2 α3
alpha	80-150	70 (7700)	Asn_{118} Complex	C-111	C-111	C-111	α3 α4
alpha	151-222	71 (7800)		C-167 C-199	C-167 M-190 C-199	C-167 M-190	α4
alpha	223-233	10 (1100)					
beta	1-71	71 (7800)	Asn_{19} Complex	C-15	C-15	C-15 (M-14 other DQ)	β1 β2
beta	72-80	8 (880)		C-79	C-79 M-78	C-79	
beta	81-93	12 (1320)			M-89		
beta	94-238	144 (15,800)		C-118 M-161 C-174 (M-182 unique) M-200	C-118 M-161 C-174 M-220	C-118 M-161 C-174 M-200	β3 β4

Fig. 4.D.1. Characteristics of postulated alpha and beta chain cleavage products. The positions of specific amino acids are indicated with one letter code and sequence number, (M-27 for methionine at position 27).

E. Phosphorylation of I_i and the Class II MHC Alpha, Beta and Chains

Experimental observations. We have demonstrated that I_i and the class II MHC alpha and beta chains can be phosphorylated. Further, it seems that either the alpha and beta chains are phosphorylated or I_i is, not all three chains at the same time. These two types of phenomena were the result of the same experimental protocol. The hypothesis that the inconsistent results was due to using a cell line (Raji) as the model system was tested, however with *S. aureus*-activated B cells the same results were seen. The hypothesis that the differing results were due to the cell density at the time of labeling was also tested, however 4 cell densities all provided one result, slight labeling of I_i with radiophosphate.

The production and use of a human antigen presenting system, with matched B and T cells, might be needed to resolve this discrepancy. In that system one would be more closely simulating the *in vivo* situation and consequently could expect more consistent results. Once the phosphorylation of these molecules are consistently seen, the residue to which the phosphate has been attached could be determined by phosphoamino acid analysis using either high voltage chromatography or a thin layer chromatography method we developed. However, since this system was unavailable at the time, I decided to pursue other, more fruitful, pathways.

Sites of phosphorylation. With the observation that phosphorylation of these molecules do occur, and with the known sequences, it is possible to hypothesize what amino acids might be phosphorylated.

The Raji cells have the haplotype of HLA-DR 3,6 (Larhammar *et al.*, 1982a; Larhammar *et al.*, 1982b; Kaufman *et al.*, 1984). The alleles of the DP and DQ molecules have not been published, however Larhammar *et al.* (1982a) published a beta chain sequence from Raji that is highly homologous (228/229 amino acids) to the DQ 1 beta chain sequence (Figueroa and Klein, 1986). For the sake of argument it will be assumed Raji has the DQ 1 allele, although for the sequence regions discussed all published alleles are homologous, unless otherwise noted.

The sequence of DR 3 alpha chain is published (Larhammar *et al.*, 1982b; Figueroa and Klein, 1986) and is shown in Fig. 4.E.1. Since (1) most phosphorylations occur on the cytoplasmic portion of the molecule; (2) only serines, tyrosines, and threonines are phosphorylated and; (3) there is only one serine in this area of the molecule; it can be hypothesized that Ser_{224} is the site of phosphorylation. This sequence area is well conserved among the published DR alpha chain sequences. Consequently, although we do not know the sequence of the DR 6 alpha chain, one can hypothesize it has a similar sequence. The DQ 1 sequence (Fig. 4.E.1) has two serines in its cytoplasmic sequence (positions 223 and 227), that are conserved in most published alleles. Only two DP alleles are published and they are entirely different from each other with respect to the amino acids

in the cytoplasmic domain that can be phosphorylated. Consequently interpretation of these isotype products is difficult.

Both the sequences of the DR 3 and DR 6 beta chains are published (Larhammar *et al.*, 1982a, Figueroa and Klein, 1986) and the cytoplasmic tails are identical except for the substitution of Phe_{236} in DR3 for Leu in DR6. In these sequences (Fig. 4.E.1) there are three sites of possible phosphorylations, two serines (229, 238) and one threonine (234). The DQ sequence from Raji cells (Larhammar *et al.*, 1982a), in Fig. 4.E.1, contains one amino acid in the cytoplasmic tail that can be phosphorylated, Ser_{224}. This serine is entirely conserved in all DQ sequences published (Figueroa and Klein, 1986). The DP beta chain sequences (Fig. 4.E.1) contain an entirely conserved serine at position 224, and four of the five published sequences have an additional serine at 231. This fifth sequence, which is very different than the other four, has a serine at position 242.

Obviously, one of the next experiments that needs to be done is to determine if all three class II MHC isotype products are equally phosphorylated. These experiments did not distinguish between the DR, DP and DQ molecules, but simply looked at the phosphorylation of all the class II MHC molecules. It is easy to imagine that these different isotype products might be phosphorylated differently. Hong *et al.* (1988) demonstrated that, in their murine system, this might be the case, as H-$2D^k$ molecules are phosphorylated while H-$2K^k$ are not. It is also interesting to speculate that the presence or absence of amino acids in the cytoplasmic tail that could be phosphorylated, in the DR, DQ, or DP molecules, might be significant in different

functions these isotypes could have.

The sequence of I_i has been published (Claesson *et al.*, 1983b), and is shown in Fig. 4.E.1. There are three serines in the cytoplasmic tail sequence (positions 9, 26 and 29). Two of these serines (positions 9 and 29) are conserved in the sequence of the murine I_i sequence (Zhu and Jones, 1989).

Since this work was done a paper was published by Spiro and Quaranta (1989) which demonstrated the phosphorylation of I_i. They showed that I_i, Ip, and p41 were phosphorylated on serine residues, but the γ2 and γ3 species were not. They were not able to define the specific residues phosphorylated nor establish a function for these modifications. Through personal communication they indicated they had similar problems to those we encountered.

Class I MHC phosphorylation precedent. The class I MHC molecule was shown to be phosphorylated on several residues. The cytoplasmic portion of HLA-A2 is shown in Fig. 4.E.1. The positions of the amino acids that can be phosphorylated are well conserved, with most being perfectly conserved. Pober *et al.* (1978) showed that the class I MHC molecules were phosphorylated *in vivo* on a serine residue on the cytoplasmic portion of the molecule and Guild and Strominger (1984a) localized that phosphorylation to serine 341, a residue conserved in all the sequences published by Figueroa and Klein (1986). Guild and Strominger (1984b) also demonstrated the phosphorylation of two serine residues *in vitro* with cAMP-dependent protein kinase, with one of

these residues in the conserved sequence Arg-Arg-Lys--Ser_{315}-Ser_{316}; the other was at position 325. Guild and Strominger (1983) further demonstrated that a cytoplasmic tyrosine residue (position 326) was phosphorylated *in vitro* by Rous sarcoma virus protein kinase. The above literature was analyzed by Guild and Strominger (1984b) to show that most of the amino acids that could be phosphorylated were phosphorylated by some kinase, (*i.e.* protein kinases *in vivo*, cAMP-dependent protein kinases, or Rous sarcoma virus protein kinase). Consequently, in the class I MHC example it seems that phosphorylations can be the result of different processes, with the specific amino acid derivatized being significant to the function involved.

Hong *et al.* (1988) demonstrated that in the murine system, only cell surface forms of H-$2D^k$ molecules were phosphorylated. R. Mittler (personal communication) has evidence that, in the human system, at least one of the phosphorylations of the class I MHC molecules might be linked to surface expression.

Hypothesized function of I_i phosphorylation. The phosphorylation of class I MHC molecules can be viewed as a similar system to the phosphorylation of class II MHC molecules. In that system phosphorylation occurs on virtually all residues on which it is possible. Further it seems that phosphorylation could occur as a result of enzymes that are involved in different functional pathways. Consequently we could hypothesize that phosphorylation could occur on

most of the residues mentioned above, and that these phosphorylations might be the result of different processes.

Phosphorylation of the adrenergic receptors (Sibley and Lefkowitz, 1985, Sibley *et al.*, 1987) could also serve as a precedent for phosphorylations of the class II MHC molecules and I_i. In that system the beta-adrenergic receptor is phosphorylated after agonist binding. The phosphorylated receptor is sequestered intracellularly and the cell is desensitized to agonist binding. Eventually the dephosphorylated receptor is returned to the cell surface and again could bind agonist.

One possible function of the phosphorylation of the class II MHC molecules could be in relation to their surface expression, as with the class I MHC molecules. Further, phosphorylation could be connected with internalization of the class II MHC molecules resulting in the attenuation of antigen presentation.

ALPHA CHAINS

219

DR 3 TM-K-G-V-R-K-S-N-A-A-E-R-R-G-P-L

DQ 1 TM-R-G-L-R-S-V-G-A-S-R-H-Q-G-P-L

BETA CHAINS

223

DR 3 TM-R-N-Q-K-G-H-S-G-L-P-P-T-G-F-L-S

DR 6 TM-R-N-Q-K-G-H-S-G-L-Q-P-T-G-L-L-S

DQ ? TM-R-S-Q-K------G-L-L-H

DP 1 TM-R-S-K-K-V-Q-R-G-S-A

li

30

TM-R-S-C-K-S-E-P-A-G-P-R-R-G-L-M-P-L-Q-E-N-N-S-I-L-D-R-Q-D-D-M

Fig. 4.E.1. Sequences of various putatively phosphorylated molecules. The sequence numbers are those from Figueroa and Klein (1986).

F. Hypothetical Scheme to Explain the Functional Significance of Structural Changes in Class II MHC Molecules and I_i.

Introduction. A scheme for the dynamic associations and cleavages of the class II MHC alpha and beta chains, and associated molecules, in the context of antigen processing and presentation, is presented in Fig. 4.F.1. The ideas of this figure are based on experiments and hypotheses.

Endoplasmic reticulum. The class II MHC alpha and beta chains can associate with I_i in the endoplasmic reticulum at the time of their synthesis, and remain with I_i during transport through the ER and Golgi (Kvist *et al.*, 1982; Claesson and Peterson, 1983a; Machamer and Cresswell, 1983). There is a large pool of I_i that is in excess of the alpha and beta chains and remains free (Kvist *et al.*, 1982; Nguyen and Humphreys, unpublished observations). Kvist *et al.* (1982) presented evidence that this pool of free I_i never leaves the ER. However, observations in this laboratory imply otherwise. Immunoprecipitates of I_i show forms of I_i processed in the Golgi (containing complex N-linked and O-linked carbohydrates), that are in excess of the alpha and beta chains (Thomas and Humphreys, unpublished observations). This finding implies that some free I_i does leave the ER.

Since all three chains (alpha, beta, I_i) have N-linked

oligosaccharide chains (Machamer and Cresswell, 1982; Claesson-Welsh *et al.*, 1986b), they must receive the asparagine-linked precursor for these carbohydrate substituents in the ER and these are trimmed to a high mannose form (M_8GN_2-Asn) before leaving this compartment.

Structural analysis of I_i has shown that a region of the molecule (Phe_{146}-His_{170}) has a high probability of forming an amphipathic alpha helix. Since class II MHC-presented peptides also have a high probability of forming such amphipathic alpha helices (DeLisi and Berzofsky, 1985), the hypothesis was developed that the I_i putative helix (146-170) might bind in the desetope formed by the alpha and beta chains. This I_i helix could block the desetope from the binding of extraneous peptides from the time of synthesis until the complex reaches a compartment containing Ig-internalized foreign antigen.

The I_i gene is also the source of the products p41, γ2 and γ3. Since these species are immunoprecipitated with antisera to the class II MHC alpha and beta chains, these species associate with the class II complex at some point.

Golgi apparatus. In this thesis I have shown that p25, a protein seen in immunoprecipitates with anti-class II MHC sera, is the C-terminal portion of a high mannose form of I_i. Although I_i has both of its N-linked carbohydrates processed to the complex form, and has an O-linked oligosaccharide chain (Machamer and Cresswell, 1982; Charron *et al.*, 1983; Machamer and Cresswell, 1984; Rudd *et al.*,

1985; Claesson-Welsh *et al.*, 1986b), these are not seen in p25 (although both N- and O-linked addition sites are included in the postulated sequence). Further, since these modifications are generally accepted to occur in the medial- or trans-Golgi (Green *et al.*, 1981), we conclude that p25 is derived from a form of I_i in the ER or cis-Golgi.

At the present time one can only speculate about the function of this cleavage. It could be a method for destruction of incomplete complexes of class II MHC molecules and associated proteins, similar to the pathway described for the degradation of incomplete T cell receptors (Lippincott-Schwartz *et al.*, 1988). Consequently the pool of I_i that undergoes this cleavage might be free I_i, dimers of I_i or I_i complexed with either the alpha or the beta chain. Another possibility is that some complexes of class II MHC proteins and associated molecules might not be considered "complete" without other components, such as p41 or I_i-CS. Consequently, complexes lacking any one of these other components might be subjected to this destructive pathway.

After some portion of the I_i molecules are degraded to p25, further processing of the oligosaccharide side chains on remaining, intact I_i, and alpha and beta chains, take place. In the medial- and trans-Golgi both of the N-linked chains on I_i are processed to complex forms (Rudd *et al.*, 1985) and the O-linked carbohydrate is added (Claesson-Welsh *et al.*, 1986b). One of the N-linked chains on the alpha chain is processed to a complex form while the other remains in the high mannose form (Shackelford and Strominger, 1983). Also, an

O-linked oligosaccharide chain is possibly added to the alpha chain (Nishikawa *et al.*, 1979; Claesson-Welsh *et al.*, 1986b). The beta chain has its N-linked chain processed to a complex form (Shackelford and Strominger, 1983).

One of the products of the I_i gene is also thought to serve as the core protein of a chondroitin sulfate proteoglycan molecule, called I_i-CS (Sant *et al.*, 1985). This large proteoglycan (up to 180 kD) is presumably manufactured in the Golgi apparatus. Most or all of these molecules may not be associated with the class II MHC proteins (Sorli and Humphreys, unpublished observations).

After processing is complete, the class II MHC proteins (with I_i, and possibly $\gamma 2$, $\gamma 3$, p41, and I_i-CS), is transported to a post-Golgi compartment.

Post-Golgi compartments. It can be hypothesized that a post-Golgi compartment is the site of the removal of I_i from the class II MHC molecules and the binding of processed antigen to the class II MHC desetope.

It is well accepted that B cells internalize and process antigen. Further, newly synthesized class II MHC molecules must pass through some post-Golgi compartment after their processing, to their eventual appearance on the cell surface. The question is whether these two compartments, the internalized antigen and the post-Golgi compartment containing newly synthesized class II MHC molecules, can fuse. Cresswell (1985) demonstrated that transferrin-neuraminidase

conjugates internalized by means of receptor-mediated endocytosis, can interact with newly synthesized class II molecules and cause desialylation of I_i and the beta chain. Consequently one can hypothesize that the compartment containing internalized antigen can fuse with the compartment containing the newly synthesized class II MHC alpha and beta chains is no evidence to indicate whether this fusion occurs before or after the foreign antigen is processed to peptides.

I_i dissociates from the class II MHC molecules after carbohydrate processing is complete (Claesson and Peterson, 1983a) and some of the best evidence implies that I_i is not present with the surface-expressed class II MHC molecules (Elliott *et al.*, 1989). If this is true, one could conclude that this dissociation takes place in some post-Golgi compartment before reaching the cell surface. Further, there is evidence for a degradative pathway of a fully processed form of I_i, that probably takes place in a post-Golgi compartment (Blum and Cresswell, 1988; Nguyen *et al.*, 1988). This pathway is detected when cells are incubated with leupeptin, and two N-terminal remnants of I_i, p21 and p10, are revealed. These peptides contain fully processed oligosaccharide chains and in pulse-chase experiments are produced maximally 2 to 5 hr after synthesis. The conclusion from these experiments is that these proteins, p21 and p10, are produced from I_i by a leupeptin-insensitive enzyme, and are normally, quickly degraded by a leupeptin-sensitive enzyme to small peptides. It can be hypothesized that this degradative pathway is significant in the removal of I_i from the class II MHC molecules in a post-Golgi

compartment, before class II MHC antigen expression on the cell surface.

It is widely accepted that class II MHC molecules bind processed foreign peptides in some site, a desetope, on their structure. The *in vitro* association rate of purified class II-presented peptides to isolated class II MHC molecules is very slow (Buus *et al.*, 1986b). This observation lead to the idea that this association might be catalyzed by accessory molecules. When cells are transfected with class II MHC genes in the presence or absence of the I_i gene, it can be shown that antigen presentation is more efficient in the presence of I_i. Since I_i is not on the cell surface, then one can conclude its effect is probably intracellular. It can then be hypothesized that I_i could be one of the principle catalytic accessory molecules.

We can now build a hypothesis how I_i might catalyze the antigen-class II MHC association based on the above observations and conclusions. As the antigen containing compartment fuses with the class II MHC containing compartment, I_i is resting in the desetope to prevent associations with random peptides, or to prevent the closure of the hydrophobic trough. When processed peptide is available, I_i is removed from the desetope as the structurally related foreign peptide is brought into the desetope in a concerted manner, which prevents closure of the hydrophobic sides of the desetope trough. The free I_i is first degraded by a leupeptin-insensitive enzyme then by a leupeptin-sensitive enzyme to small peptides that do not compete further for desetope binding. According to this hypothesis one might expect that purified class II

MHC molecules, without the catalytic effect of I_i leaving, would be only slowly charged with amphipathic foreign peptides, as has been observed experimentally (Buus *et al.*, 1986b). Further this hypothesis is consistent with the observations that chloroquine, which neutralizes intracellular acidic compartments, inhibits class II MHC antigen presentation (Grey and Chestnut, 1985), I_i dissociation from the alpha and beta chains (Nowell and Quaranta 1985, and the production of these peptides in the presence of leupeptin (Blum and Cresswell, 1988).

Kelner and Cresswell (1986) have found complexes of class II MHC proteins and associated molecules consisting of alpha and beta chains with I_i and I_i-CS in a 1:1:1:1 ratio. If I_i is not present on the cell surface, then it can be concluded that I_i-CS associates with some class II MHC molecules intracellularly. Sant *et al.* (1985b) previously demonstrated that only 2-5% of class II complexes had I_i-CS, including some cell surface molecules, and that this interaction was rapid and short-lived. Consequently it can be hypothesized that after I_i-CS associates with the class II MHC molecules with bound I_i, I_i dissociates, the complex goes to the cell surface and I_i-CS dissociates. The questions are still unanswered whether there are additional molecules associated with the surface-expressed class II MHC molecules, as $\gamma 2$, $\gamma 3$ or p41.

Surface expression and reinternalization. Class II MHC molecules containing processed peptide are expressed on the cell surface, for

presentation to T cells. It has been shown that some class I MHC molecules are phosphorylated when surface-expressed. Since I have shown in this thesis that the class II MHC alpha and beta chains, along with I_i, can be phosphorylated, that phosphorylation might occur when the class II complex is surface-expressed. This change could be a signal for the internalization of the complex, or might be a mechanism for attenuation of presentation. Specifically, the phosphorylation might be a feedback signal indicating that effective presentation has occurred and that the surface expression of the complex is no longer needed. Alternatively, binding of soluble factors to the B cell, that initiate proliferation and differentiation, also could cause phosphorylation of the class II MHC molecules and associated proteins and thereby terminate surface expression and antigen presentation.

It has been shown in this thesis that the class II MHC molecules might undergo proteolytic cleavages. The putative sites of these cleavages are located around the desetope and cleavages at these locations could terminate the antigen presenting capability of the class II MHC molecules. It can be imagined that after presentation of antigen to T cells, there would be a point in time when it would be beneficial to terminate any presentation. These proteolytic cleavages could serve in this regard.

Proteolytic cleavages of the alpha and beta chains might occur while foreign antigen is present, after the complex has reached the cell surface. This would serve to prevent further antigen presentation to T cells after sufficient presentation might have occurred.

Alternatively, these sites could be accessible for cleavage by extracellular proteases only after release of foreign peptide. That cleaved peptide binding site would be unable to adsorb, and thus present, ambient peptides. Proteases are known to be released by activated T cells (Pasternack and Eisen, 1985; Pasternack *et al.*, 1986). After internalization, proteolytic cleavage of the alpha and beta chains could also occur to inactivate the class II MHC presentation ability. This could serve as a mechanism to destroy complexes that have presented foreign antigen. Furthermore, if I_i is required to catalyze foreign peptide binding to class II MHC molecules, these recycled surface molecules, void of I_i, might bind such peptides inefficiently.

The studies presented in this thesis have characterized some structural changes in I_i and the class II MHC alpha and beta chains, which might be important in the regulation of antigen presentation. With those, and information from other laboratories, I have put together the hypothetical scheme above. Much work is needed to test the hypotheses it contains. However, an understanding of the control of antigen processing and presentation will be valuable in the research of a large number of biological problems.

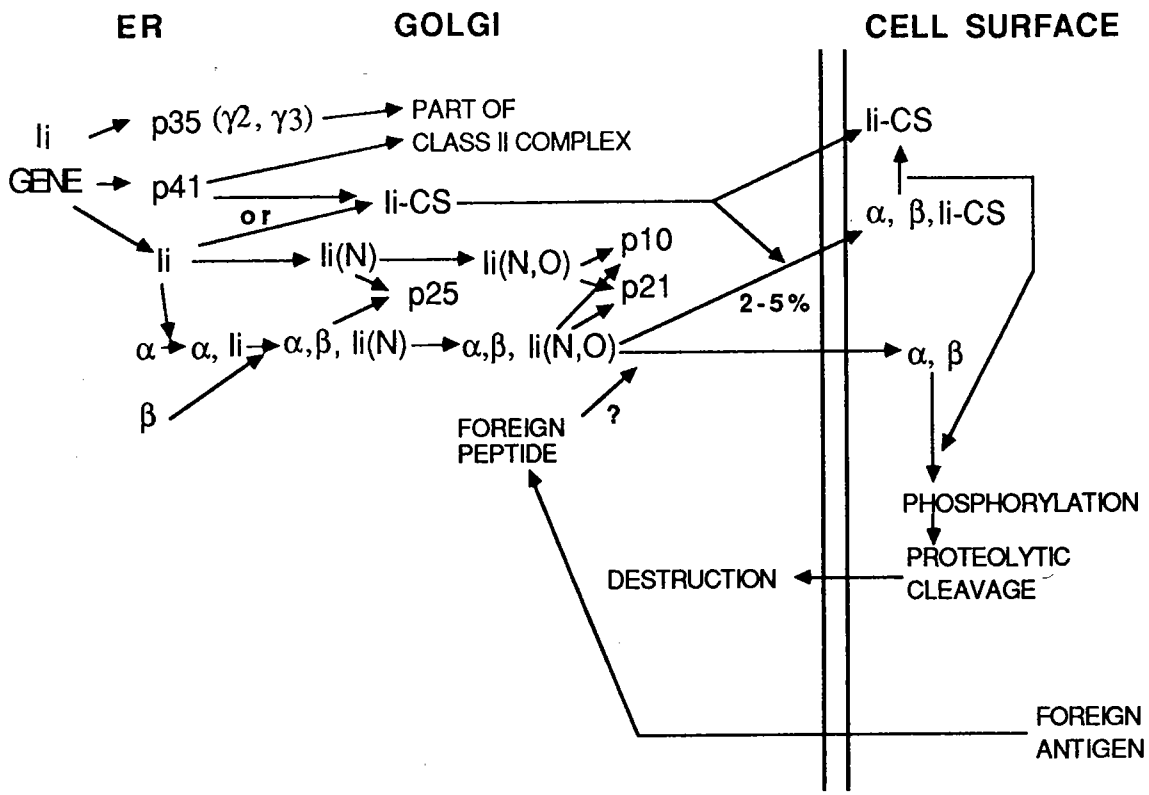


Fig. 4.F.1. Hypothetical scheme to explain the functional significance of structural changes in class II MHC molecules and I_i, and the associations of these molecules, in the context of antigen processing and presentation.

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